Quantification of DNA and hemoglobin adducts of 3,4-epoxy-1,2-butanediol in rodents exposed to 3-butene-1,2-diol

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1,3-Butadiene (BD) is a confirmed rodent carcinogen and a suspect human carcinogen that forms mutagenic epoxide metabolites during biotransformation. Species differences in the roles of individual DNA reactive intermediates in BD mutagenicity and carcinogenicity are not completely understood. Evidence suggests that 1,2:3,4-diepoxybutane (DEB) is responsible for the mutagenic effect induced by exposures to low concentrations of BD in mice and that metabolites of 3-butene-1,2-diol (BD-diol) are involved in the mutagenicity at high exposures in both mice and rats. Two reactive metabolites, 3,4-epoxy-1,2-butanediol (EB-diol) and hydroxymethylvinyl ketone (HMVK), are formed during the biotransformation of BD-diol and could potentially be involved in BD-diol associated mutagenicity. To examine the role of EB-diol in BD-diol mutagenicity we have evaluated the dosimetry of N7-(2,3,4-trihydroxybutyl)guanine (THB-Gua) and N-(2,3,4-trihydroxybutyl)valine (THB-Val) in female B6C3F1 mice and female F344 rats exposed by inhalation to 0, 6, 18 and 36 p.p.m. BD-diol for 4 weeks (6 h/day × 5 days/week).

Results showed higher levels of both THB-Gua and THB-Val in mice than in rats. An evaluation of THB-Gua adducts showed virtually no differences between liver and lung for either species, suggesting that EB-diol is stable and is freely circulated. The data also indicated that THB adduct formation began to plateau around 18 p.p.m. in both species. Most importantly, the shape of the dose-response curve for THB adduct formation mimicked the one observed for hypoxanthine-guanine phosphoribosyltransferase (Hprt) mutation frequency. This showed that THB adducts, which are not thought to be responsible for causing the mutations, are good quantitative indicators of mutagenicity in rodent exposed to BD-diol. Although the potential contribution of HMVK still needs to be evaluated, the data suggest that EB-diol is responsible, at least in part, for BD-diol associated mutagenicity in rodents.

Introduction

1,3-Butadiene (BD) is an important commercial chemical and environmental pollutant that causes exposure-related increases of various cancers in mice and rats. Mice are the more sensitive species and females the more sensitive sex (1–5). Female B6C3F1 mice exposed via inhalation to BD concentrations as low as 6.25 p.p.m. developed lung tumors (2). Although different interpretations of the results exist, epidemiology studies show an increase in the incidence of leukemia in synthetic rubber production workers exposed to BD (6) and an increase in lymphohematopoietic cancers in BD production workers (7). The National Toxicology Program classifies BD as a known human carcinogen (8), the International Agency for the Research of Cancer classifies BD as a probable human carcinogen (9) and BD is classified by the US Environmental Protection Agency as carcinogenic to humans by inhalation (10).

BD biotransformation is complex and involves several pathways. Of particular interest in the current study is the 3-butene-1,2-diol (BD-diol) metabolic pathway (Scheme 1). BD is initially metabolized via cytochrome P450 (CYP450) to 1,2-epoxy-3-butene (EB) (11–13). BD-diol is then formed by the subsequent hydrolysis of EB by epoxide hydrolase (EH) (11–14). BD-diol is metabolized by CYP450 to form hydroxymethylvinyl ketone (HMVK) (15) or 3,4-epoxy-1,2-butanediol (EB-diol) (11,13,16). BD-diol can also be metabolized by alcohol dehydrogenase (ADH) to 2-hydroxy-3-butenal (HBAL), which may spontaneously rearrange to form HMVK (17). HMVK reacts with glutathione via glutathione-3-transferase (GST) to eventually form mercapturic acids (18–22) including 1,2-dihydroxy-4-(N-acetylcysteiny)butane (MI), the primary mercapturic acid metabolite found in the urine of humans exposed to BD (19).

Abbreviations: AAG, alkyladenine glycosylase; ADH, alcohol dehydrogenase; BD, 1,3-butadiene; BD-diol, 3-butene-1,2-diol; CYP450, cytochrome P450; DEB, 1,2:3,4-diepoxybutane; EB, 1,2-epoxy-3-butene; EB-diol, 3,4-epoxy-1,2-butanediol; EH, epoxide hydrolase; GC, gas chromatography; GC-MS/MS, gas chromatography–tandem mass spectrometry; GST, glutathione-3-transferase; HBAL, 2-hydroxy-3-butenal; HMVK, hydroxymethylvinyl ketone; Hprt, hypoxanthine-guanine phosphoribosyltransferase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MI, 1,2-dihydroxy-4-(N-acetylcysteiny)butane; PFPITC, pentafluorophenylisothiocyanate; SRM, selected reaction monitoring; THB-Gua, N7-(2,3,4-trihydroxybutyl)guanine; THB-Val, N-(2,3,4-trihydroxybutyl)valine.

Scheme 1. BD-diol metabolism.
Several reactive intermediates are formed during BD metabolism. Although DEB, EB and EB-diol are known mutagens (23,24), the role of these epoxides in BD mutagenesis and carcinogenesis is not fully understood. By evaluating the induction of Hprt mutations in rodents exposed to relevant concentrations of BD, EB, DEB and BD-diol, Walker et al. (25) proposed that DEB is responsible for mutagenicity in mice exposed to low levels of BD, while metabolites of BD-diol play a significant role in the mutagenic response in mice and rats exposed to higher concentrations of BD. EB is not thought to play a major role in mutagenicity in either species (25,26).

In addition to being implicated in the mutagenicity of BD, the BD-diol pathway is important for several reasons. First, exposure to BD-diol via inhalation causes an increase in Hprt mutation frequency in both mice and rats (25). Second, intraperitoneal administration of a high dose of BD-diol is hepatotoxic in rats (27). Third, BD-diol is a precursor to MI, an important urinary metabolite in humans exposed to BD (19). Fourth, BD-diol is a major metabolite of BD in single-pass liver perfusion experiments conducted in both mice and rats (28) and is present in the urine of rodents exposed to BD (25,29). Finally, as previously shown, the metabolism of BD-diol yields two reactive compounds. EB-diol is a known mutagen (23,24), while HMVK is expected to be mutagenic (25,29). Finally, as previously shown, the metabolism of BD-diol exposed mice and rats to BD-diol and the formation of an 87-guanine DNA adduct and an N-terminal valine adduct of hemoglobin derived from the in vivo conversion of BD-diol to EB-diol. This objective was accomplished by quantifying N7-(2,3,4-trihydroxybutyl)guanine (THB-Gua; Figure 1a) and N-(2,3,4-trihydroxybutyl)valine (THB-Val; Figure 1b) in female B6C3F1 mice and female F344 rats exposed via inhalation to 0, 6, 18 and 36 p.p.m. BD-diol (6 h/day × 5 days/week × 4 weeks). In this fashion, we evaluated tissue and species-specific differences in DNA adduct formation in liver (a major site of metabolism) and lung (a target organ for BD-induced cancer in the mouse) of BD-diol exposed mice and rats. Species differences were also evaluated for hemoglobin adducts. In addition, DNA and hemoglobin adduct formation were compared with the Hprt mutant frequency data for concurrently exposed rodents to understand the role of EB-diol in mutagenicity occurring via the BD-diol pathway.

**Fig. 1.** (a) THB-Gua and (b) THB-Val.

**Materials and methods**

**Chemicals**

Lysis buffer, phenol–H2O–chloroform and Proteinase K were purchased from Applied Biosystems (Foster City, CA); RNase A from Sigma Aldrich (St Louis, MO); pentafluorophenylisothiocyanate (PFPTIC) from Fluka (Milwaukee, WI); ± BD-diol from Aldrich Chemical (Milwaukee). All other chemicals were purchased from common vendors.

**Animals and BD-diol exposures**

The exposure protocol has been presented in detail elsewhere (25). Briefly, 4–5 week old female B6C3F1 mice and female F344 rats were exposed via inhalation to various concentrations of BD-diol vapor using whole-body exposure chambers (6 h/day × 5 days/week × 4 weeks). Two separate series of exposures were performed using an identical protocol. In the first series, rodents were exposed to 0, 6 or 18 p.p.m. BD-diol, whereas in the second series another group of rodents was exposed to 0 or 36 p.p.m. BD-diol. BD-diol exposure atmospheres were monitored using two techniques, a real-time hydrocarbon analyzer and an integrated sample to verify the response of the hydrocarbon analyzer relative to actual BD-diol concentrations. The hydrocarbon analyzer was a real-time flame ionization detector (Model 300H, Analytical Instruments, Irvine, CA) calibrated against a certified propane standard. This instrument gave real-time data for maintaining the stability of the exposure concentrations and for assessing the rise and decline of BD-diol chamber concentrations. This set of BD-diol densitograms were collected in 30 ml glass impingers containing 20 ml of purified H2O. Impingers were placed on ice and samples were collected at a flow rate of 0.3 liters per min for 30 min. Periodically, back-up impingers were collected to verify the absence of sample breakthrough in the front trap. After collection, the samples were immediately analyzed by gas chromatography/flame ionization detection. Stock standards were made from aliquots of BD-diol purchased from animal pharmaceutical suppliers, and calibration solutions were created in ddH2O and spanned from 7.8–125 μg/ml. BD-diol atmospheres were compared with five-point calibration curves that spanned the exposure range required to measure with accuracy the lower and upper ends of the BD-diol exposure concentrations used in rodent inhalation studies. At no time did the BD-diol exposure atmosphere concentration exceed the upper calibration concentration. Analysis of exposure atmospheres by GC and a flame ionization detector revealed no detectable impurities, attesting to both the stability of the exposure atmosphere and the lack of detectable impurities in the reference BD-diol (certified at >99% pure). Actual concentrations of BD-diol were 6.0 ± 0.7, 16.5 ± 0.7 and 33.4 ± 4.7 p.p.m., corresponding to nominal concentrations of 6, 18 and 36 p.p.m. Blood and tissues were collected within 2 h after the cessation of final BD-diol exposure.

**DNA isolation**

Note: centrifugation steps were performed at 4°C. Approximately 0.5 g tissue was weighed and homogenized in 8 ml cold phosphate buffered saline (PBS) buffer (1×) (if smaller amounts of tissue were used, reagent volumes were adjusted accordingly). This homogenate was transferred to a 50 ml sample tube and centrifuged for 10 min at 1500 g. After discarding the supernatant, the pellet was resuspended in 1 ml of 70% ethanol and the mixture centrifuged for 30 min and subsequently cooled on ice. The supernatant was discarded, the pellet resuspended in 0.5 ml of 100% ethanol and the extraction repeated. After transferring the top phase to a new 50 ml sample tube, 5 ml of 10% SDS was added, and the solution mixed by vortexing. The solution was centrifuged at 18000g for 30 min, and the supernatant discarded. To the pellet, 1 ml of ddH2O was added, and the solution mixed by vortexing. The solution was centrifuged at 18000g for 5 min at 18000 g. DNA from the reference BD-diol (certified as >99% pure). Actual concentrations of BD-diol were 6.0 ± 0.7, 16.5 ± 0.7 and 33.4 ± 4.7 p.p.m., corresponding to nominal concentrations of 6, 18 and 36 p.p.m. Blood and tissues were collected within 2 h after the cessation of final BD-diol exposure.

**DNA quantification**

DNA (250–500 ng) was precipitated by adding 300 μl of NaCl (5 M) and 10 ml of 100% ethanol followed by gentle mixing. The solution was centrifuged for 10 min at 18000 g and the supernatant discarded. To the pellet, 10 μl of 70% ethanol was added, centrifugation repeated, and the supernatant discarded. The pellet was resuspended in 4 ml of PBS (1×) and rotated overnight at 4°C and 10 μl of RNase A (1.3 U) was added, and the solution incubated for 30 min at 37°C. To this, 1 ml of ddH2O and 6.5 ml of 100% ethanol were added, the solution was then mixed and centrifuged for 10 min at 18000 g. The supernatant was discarded, the pellet resuspended in 10 ml of 70% ethanol, and the mixture centrifuged for 10 min at 18000 g. After removing the supernatant, the pellet was resuspended in 0.5 ml of ddH2O, placed at 4°C for 1 h and then stored until use at –80°C. A small aliquot was used for the initial determination of DNA concentration by UV spectrophotometry. Final DNA concentration was determined using a PicoGreen dsDNA quantification kit (Molecular Probes, Eugene, OR).

**THB-Gua sample preparation**

DNA (250–500 μg as determined by initial measurements using UV spectrophotometry) and internal standard [13C4]-THB-Gua were combined in a sample tube and brought to a final volume of 1 ml with HPLC grade H2O. The sample was incubated at ~95°C for 30 min and subsequently cooled on ice.
for 5 min. To the sample 1 ml of HPLC grade H2O was added and the mixture filtered by centrifugation for 2 h at 5000 g using a Centricon-10 filter. Following filtration, the sample was dried by centrifugal lyophilization and redissolved in 50 μl of HPLC grade H2O. The sample was then transferred to a sample vial and stored at −80°C until analysis.

**Quantification of THB-Gua**

Samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a Finnigan Surveyor micro-LC system coupled with a Finnigan TSQ Quantum triple-quadrupole mass spectrometer (Thermo Electron, San Jose, CA). Separation of THB-Gua from unmodified bases was performed on an Aquasil C18 column (5 μm; 2.1 mm × 150 mm) (Thermo Electron) preceded by a guard cartridge, using HPLC grade H2O with 0.1% acetic acid (solvent A) and acetonitrile with 0.1% acetic acid (solvent B). Injection volumes of 10 μl were used. The following gradient was used: 0–1 min, 100% A; 1–10 min, 100–85% A; 10–15 min, 85–20% A; 15–25 min, 100% A. The flowrate throughout the analysis was 200 μl/min. MS parameters were as follows: polarity, positive; spray voltage, 4500 V; sheath gas, 47 (arbitrary unit); auxiliary gas, 5 (arbitrary unit); capillary temp, 350°C; source CID, 10 V; collision energy, 22 V; collision gas, 1.5 mTorr. Adducts were detected using MS/MS using selected reaction monitoring (SRM) of m/z 256→152, corresponding to the loss of the THB side-chain (32). The ratio of the analyte peak area to that of the internal standard, measured by SRM of m/z 260→156, was used to quantify the adduct.

**Globin isolation**

Note: centrifugation steps were performed at 4°C. Globin was isolated by the method of Mower et al. (33), with slight modifications for the isolation of mouse globin (34). Packed red blood cells were mixed and lysed with 1 ml ddH2O. After complete mixing, acid propanol (9 ml for mouse globin and 6 ml for rat globin) was added and the solution vortexed. The solution was then centrifuged for 30 min at 1500 g. The supernatant was carefully transferred into a fresh tube, ethyl acetate added (6 ml for mouse and 4 ml for rat) and the solution vortexed until the globin precipitated. The sample was then centrifuged for 5 min at 1500 g and the supernatant discarded. The pellet was resuspended in 4 ml ethyl acetate and the centrifugation step repeated. These steps were repeated until a colorless supernatant was obtained. The step was then repeated using n-pentane. The resulting pellet was dried and stored at −80°C.

**THB-Val sample preparation**

The globin was derivatized based on a modified Edman degradation (35). For this, 2–5 mg mouse globin or 6–10 mg rat globin was dissolved in 1.5 ml formamide and 30 μl of 1 M NaOH added. To this, 20 μl of PFPTC was added and the reaction mixture incubated at room temperature overnight with shaking. Derivatized THB-Val internal standard [THB-(13C2)Val-PFPPTH] was added and the samples incubated for an additional 1.5 h at 45°C. The sample was extracted 3 times with 3 ml diethylether and dried by centrifugal lyophilization. The residue was resuspended in 1 ml of 0.1 M Na2CO3 and applied to a 200 mg Extract-Clean C18 solid phase extraction column (Alltech Associates, Deerfield, IL) pre-equilibrated with 50:50 formamide:H2O (v/v). After sample application, the column was washed with 1 ml of ddH2O and the adduct containing fraction eluted with 3 ml acetonitrile prior to being dried by centrifugal lyophilization. The sample was extracted by adding 1 ml of 60% methanol and 2 ml of hexane. The aqueous layer was removed and dried by centrifugal lyophilization prior to acetylation by adding 188 μl acetonitrile, 32.5 μl triethylamine, 32.5 μl acetic anhydride and incubating for 30 min at room temperature. It was subsequently dried by centrifugal lyophilization, resuspended in 3 ml of n-pentane and washed with 2 ml of 60% methanol. The n-pentane layer was evaporated, the sample resuspended in 50 μl toluene, placed in a sample vial and stored at −80°C until analysis.

**THB-Val quantification**

Samples were analyzed by GC–tandem mass spectrometry (GC-MS/MS) using a Trace GC2000 coupled with a Finnigan TSQ 7000 triple-quadrupole mass spectrometer (Thermo Quest Finnigan, San Jose, CA). The analysis was performed with electron-capture negative chemical ionization (ECNI) using methane as the reagent gas (3 × 10−5 mbar). Sample volumes of 2 μl were injected by an AS2000 autosampler (Thermo Quest Finnigan) in the splitless mode at a 270°C injector temperature. Separation was performed on an Alltech EC-5 column (30 μm × 0.32 mm, 1.0 μm film thickness (Alltech Associates) with 10 μl helium head pressure. The capillary column temperature was programmed to increase from 100°C to 320°C at a rate of 10°C/min. The TSQ 7000 was operated using the following parameters: polarity, negative; electron energy, 70 V; source pressure, 3.5 Torr; ion source temperature, 150°C; emission current, 500 μA; collision energy, 10 V; collision gas, 2.5 mTorr. Adducts were measured by MS/MS using SRM of m/z 534→303, m/z 534 corresponds to the loss of [HF] from the derivatized adduct and m/z 303 corresponds to the loss of the THB side-chain from the adduct (36). Adduct quantification was performed by comparing analyte peak area with that of the internal standard (SRM of m/z 539→308).

**Results**

**Dosimetry of THB-Gua**

Because of the stereochemical characteristics of EB-diol, THB-Gua exists as two racemates and two meso isomers (37). An LC-MS/MS chromatogram from the liver of female F344 rats exposed to 36 p.p.m. BD-diol clearly shows a peak containing the racemic mixture of enantiomers as well as an overlapping peak corresponding to meso THB-Gua (Figure 2). Although racemic THB-Gua internal standard was used, the levels of racemic and meso adduct were combined and presented as total THB-Gua. The limit of detection, defined as the amount of standard yielding a signal/noise (S/N) of ≥3, was ~3.5 fmol of pure standard injected on column. A representative calibration curve is provided in Figure 3. The smallest amount of adduct measured in samples from BD-diol exposed rodents was ~33 fmol injected on column.

Summary data for THB-Gua formation in rodents exposed to BD-diol is presented in Table I. Several statistical comparisons were performed with these data using Student’s t-test (α = 0.05). A comparison to determine if a statistically significant difference in mean adduct levels existed between species showed that mice have a greater amount of adduct than rat in...
both the liver and the lung at 6 and 18 p.p.m. BD-diol. A 2.4-fold greater amount of THB-Gua was present in mouse liver and a 2.3-fold increase in mouse lung was observed for rodents exposed to 6 p.p.m. BD-diol, whereas at 18 p.p.m. a 2.3-fold increase existed for mouse liver and 3.5-fold increase for mouse lung.

A second comparison was performed to determine if there were tissue differences in the levels of adducts. Our data showed only one instance of a statistical difference between tissues, with significantly higher levels of adducts found in liver than lung of rats exposed to 18 p.p.m. BD-diol. Although the apparent difference was statistically significant, it appears to be largely a function of the small SD and only represents an 18.5% difference between mean adduct levels in liver and lung.

Figure 4 shows the dose–response curves for THB-Gua formation in liver from mice and rats. Adduct formation was almost linear in mice from 0 to 18 p.p.m. BD-diol. The efficiency of adduct formation, defined as the amount of THB-Gua/ppm BD-diol, decreased as the exposure increased from 6 to 18 p.p.m. BD-diol. Due to a technical error, data are unavailable for THB-Gua levels in mice exposed to 36 p.p.m. BD-diol. In the rat, THB-Gua formation began to plateau at the higher exposure concentrations. As was observed in the mouse, efficiency also decreased as the exposure level increased.

Dosimetry of THB-Val

The different stereoisomers of THB-Val are observed in the GC-MS/MS chromatogram from a female B6C3F1 mouse exposed to 36 p.p.m. BD-diol (Figure 5). The two overlapping peaks correspond to the meso forms of the adduct, while the racemates coelute. As with THB-Gua, a combination of isomers was used for quantification. The limit of detection (S/N ≥ 3) was ~200 amol of pure standard injected on column. A representative calibration curve is provided in Figure 6. The smallest amount of adduct measured in globin from BD-diol exposed rodents was ~40 fmol injected on column.

Table II presents the summary data for THB-Val formation in the BD-diol exposed mice and rats. Comparisons of mean adduct levels between species were identical to what was observed for THB-Gua. Mice formed a greater amount of adduct/mg globin as demonstrated by a statistically significant difference in adduct levels between species at 6, 18 and 36 p.p.m. BD-diol. There was a 2.1-fold, 2.0-fold and 1.7-fold increase in the amount of THB-Val measured in mice and rat at 6, 18 and 36 p.p.m. BD-diol, respectively. In both species, there was a quantifiable level of adduct in globin from control animals. Control mice had a 1.4-fold greater amount of adduct than control rats.
Rat lung as well as kidney from both rats and mice exposed to et al differences between liver and lung for either species. Koc mutant frequencies in splenic T-cells of mice were confusate following single-pass liver perfusion experiments (28). Several studies have demonstrated that mice have higher levels of BD metabolites than rats in tissues (43,44) and blood (36,37) also observed similar THB-Gua levels in liver, lung, and kidney but much higher in testis of male Aag mice (49). This finding suggests that the testis has a greater ability to remove adducts via base excision repair at exposure levels that are unlikely to saturate DNA repair. Since the results in our study show identical levels of THB-Gua between tissues with similar repair capabilities, it appears EB-diol is stable enough to circulate rapidly and evenly throughout the body as has been previously proposed (37). However, to definitively determine the biological stability of EB-diol a method to quantify this metabolite needs to be developed.

The shape of the dose–response curves for THB-Gua and THB-Val are also noteworthy. Although no DNA adduct data for mice exposed to 36 p.p.m. BD-diol is available, the increase in THB adduct levels was similar when the BD-diol exposure increased from 6 to 18 p.p.m. THB-Gua in liver increased by 2.1-fold and THB-Val by 2.4-fold. Because of the extended dose–response data in the rat, the similarities in THB adduct formation are even more evident. The amount of adduct increased 2.1-fold for THB-Gua in liver and 2.5-fold for THB-Val when the exposure concentration was increased from 6 to 18 p.p.m. BD-diol. When the exposure was increased from 18 to 36 p.p.m. BD-diol, THB-Gua in the liver changed 1.6-fold and THB-Val changed 1.4-fold. Overall, at the exposure levels used in the current study THB adduct formation began to plateau at ~18 p.p.m. BD-diol, indicative of metabolic saturation for EB-diol formation. This is not surprising since BD-diol clearance in female mice exposed to BD-diol is supralinear (25), which also indicates metabolic saturation.

In order to compare our results with previous studies, it is important to understand the relevance of the selected BD-diol concentrations. A single 6 h exposure to 18 p.p.m. BD-diol in female B6C3F1 mice or female F344 rats yielded an equivalent plasma concentration of BD-diol as a single 6 h exposure to 200 p.p.m. BD. In female B6C3F1 mice, exposure to 36 p.p.m. BD-diol yielded ~25% of the plasma concentration attained following exposure to 625 p.p.m. BD (25). Previous studies showed THB-Gua formation did not reach saturation in mice following exposures up to 1300 p.p.m. BD (37,50), while in rats THB-Gua formation began to plateau at BD exposures >62.5 p.p.m. (37). Our results showed that THB-Gua formation in rats began to plateau ~18 p.p.m. BD-diol, corresponding to a BD exposure of 200 p.p.m. We do not have dose–response data for THB-Gua formation in mouse. At lower concentrations our hemoglobin data were also consistent with the published results showing THB-Val formation did not reach saturation in male B6C3F1 mice and male Sprague–Dawley rats following 2 weeks of exposure to concentrations up to 20 p.p.m. BD (40), or in female B6C3F1 mice or female F344 rats exposed to concentrations up to 62.5 p.p.m. BD for 4 weeks (41). At higher exposures THB-Val formation peaked 20, 62.5 and 625 p.p.m. BD. While THB-Gua levels in these tissues appear comparable, adduct levels in testis may be different. Booth et al. (39) showed that liver and lung have similar levels of THB-Gua, but the testis had lower levels in mice and rats exposed to 1, 5 or 20 p.p.m. BD. On the other hand, mice exposed to 200 p.p.m. BD had similar levels of THB-Gua in liver, lung and testis (38). The differences between THB-Gua adduct levels in testis and the other tissues may be owing to the variations in DNA repair capacity at different BD exposure levels. The activity of alkyladenine glycosylase (Aag), an enzyme involved in repairing N7-methylguanine adducts in mice (48), is similar in liver, lung, and kidney but much higher in testis of male Aag mice (49).

A graphical presentation of the data (Figure 7) shows the dose–response in both species. For both rats and mice, THB-Val formation began to plateau at the higher concentrations. As was observed for THB-Gua, adduct efficiency decreased as the exposures increased.

### Discussion

When comparing the THB-adduct formation between species in the current study, we showed that mice exposed to BD-diol form greater amounts of both THB-Gua and THB-Val than rats. This species difference agrees with the existing data showing that mice form greater amounts of EB-diol derived DNA and hemoglobin adducts than rats exposed to a range of BD from 1 to 1250 p.p.m. (37–42). The differences in THB adduct formation between mice and rats reflect species differences observed in the metabolism and toxic effects of BD. Several studies have demonstrated that mice have higher levels of BD metabolites than rats in tissues (43,44) and blood (43,45) following in vivo exposure to BD, as well as in perfusate following single-pass liver perfusion experiments (28). Hprt mutant frequencies in splenic T-cells of mice were consistently greater than those observed in rats even at relatively low concentrations of BD. THB adduct dosimetry in female B6C3F1 mice and female F344 rats exposed to BD-diol

Table II. THB-Val dosimetry in female B6C3F1 mice and female F344 rats exposed to BD-diol

<table>
<thead>
<tr>
<th>Exposure</th>
<th>n</th>
<th>THB-Val</th>
<th>THB-Val/p.p.m. BD-diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>0.07 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>12.3 ± 2.2</td>
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</tr>
<tr>
<td>18</td>
<td>3</td>
<td>29.2 ± 1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>35.8 ± 1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>0.05 ± 0.003</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>5.84 ± 1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>14.7 ± 1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>20.9 ± 1.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD with units of pmol adduct/mg globin. **Statistically significant difference between species at this exposure concentration.

![Graph of THB-Val dosimetry](https://example.com/graph.png)

Fig. 7. THB-Val dose–response in female B6C3F1 mice and female F344 rats.

A graphical presentation of the data (Figure 7) shows the dose–response in both species. For both rats and mice, THB-Val formation began to plateau at the higher concentrations. As was observed for THB-Gua, adduct efficiency decreased as the exposures increased.

### Discussion

When comparing the THB-adduct formation between species in the current study, we showed that mice exposed to BD-diol form greater amounts of both THB-Gua and THB-Val than rats. This species difference agrees with the existing data showing that mice form greater amounts of EB-diol derived DNA and hemoglobin adducts than rats exposed to a range of BD from 1 to 1250 p.p.m. (37–42). The differences in THB adduct formation between mice and rats reflect species differences observed in the metabolism and toxic effects of BD. Several studies have demonstrated that mice have higher levels of BD metabolites than rats in tissues (43,44) and blood (43,45) following in vivo exposure to BD, as well as in perfusate following single-pass liver perfusion experiments (28). Hprt mutant frequencies in splenic T-cells of mice were consistently greater than those observed in rats even at relatively low concentrations of BD. THB adduct dosimetry in female B6C3F1 mice and female F344 rats exposed to BD-diol.
in male Wistar rats at 200 p.p.m. BD (51), corresponding to an exposure of 18 p.p.m. BD-diol. Direct comparisons between BD and BD-diol are complicated, however, by the presence of different P450 substrates and their potential effects on metabolism.

Of great interest are the similar shaped dose–response curves observed for THB adduct formation and the induction of Hprt mutant frequency in splenic T-cells from mice (Figure 8a) and rats (Figures 8b and 9) exposed to BD-diol. These similarities suggest that THB adducts are good quantitative indicators of EB-diol formation and its resulting splenic T-cell mutagenicity at the Hprt locus in rodents exposed to BD-diol. A similar conclusion was given by Meng et al. (52) who found that THB-Gua formation correlated with Hprt mutation frequency in rodents exposed to 0, 6.25, 62.5 and 625 p.p.m. BD. Based on both the current study and the previous work, it appears that EB-diol plays a significant role in BD-diol and BD associated mutagenicity. It is not likely that THB-Gua is actually causing the Hprt mutations since N-7 alkylguanine adducts are not promutagenic. Other minor promutagenic THB adducts may, however, be responsible. This will require additional studies, as these adducts have not been quantified.

In summary, this is the first description of THB adduct dosimetry in rodents exposed to BD-diol. The data agree with the species differences observed in BD metabolism, mutagenicity and carcinogenicity. More importantly, our data show that THB adducts arising from EB-diol may be good quantitative indicators of mutagenicity. While our results suggest that EB-diol contributes to BD-diol associated mutagenicity, it remains necessary to determine the contributions made by HMVK. Sprague and Elfarra (22) showed that as the dose was increased the percentage of BD-diol metabolized to HMVK-derived mercapturic acid decreased. The authors interpret this as an indication of metabolic saturation. However, the formation of HMVK-derived mercapturic acid metabolites appears to be approximately linear up to doses of 1420 µmol/kg or 125 mg/kg, in male B6C3F1 mice and male Sprague–Dawley rats. Following intraperitoneal injection of 100 mg/kg BD-diol, a plasma concentration >100 μg BD-diol/ml in male B6C3F1 mice was observed (16) while inhalation of 36 p.p.m. BD-diol for 6 h, the highest concentration used in our current study, produced a plasma concentration of ~2.5 μg BD-diol/ml in female B6C3F1 mice. Therefore, it appears as if HMVK formation, indicated by HMVK-derived mercapturic acid formation, is linear at plasma BD-diol concentrations more than 40 × greater than that evaluated in the current study. Because Walker et al. (25) observed a plateau in the induction of Hprt mutations at concentrations considerably lower than where HMVK saturation would occur, it is unlikely that HMVK is as important as EB-diol in BD-diol associated mutagenicity. However, empirical evidence is needed to prove this hypothesis. While the potential mutagenic activity of HMVK is yet to be determined, it is likely that EB-diol is responsible, at least in part, for the mutagenicity associated with BD-diol.

Acknowledgements

This research was funded by the HEI (99–5), the NIEHS (R01-ES012689, R42-ES11746, and P30-ES10126), and the NCI (P30-CA16086). Support was also provided by an individual NRSA postdoctoral fellowship from the NIEHS (F32-ES012357–01).

Conflict of Interest Statement: The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS.

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Received December 16, 2004; revised April 18, 2005; accepted May 3, 2005