

Evaluation of Cancer Tests of 1,3-Butadiene Using Internal Dose, Genotoxic Potency, and a Multiplicative Risk Model

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Abstract

In cancer tests with 1,3-butadiene (BD), the mouse is much more sensitive than the rat. This is considered to be related to the metabolism of BD to the epoxide metabolites, 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane, and 1,2-epoxy-3,4-butanediol. This study evaluates whether the large difference in outcome in cancer tests with BD could be predicted quantitatively on the basis of the concentration over time in blood (AUC) of the epoxide metabolites, their mutagenic potency, and a multiplicative cancer risk model, which has earlier been used for ionizing radiation. Published data on hemoglobin adduct levels from inhalation experiments with BD were used for the estimation of the AUC of the epoxide metabolites in the cancer tests. The estimated AUC of the epoxides were then weighed together to a total genotoxic dose, by using the relative genotoxic potency of the respective epoxide inferred from *in vitro* hprt mutation assays using EB as standard. The tumor incidences predicted with the risk model on the basis of the total genotoxic dose correlated well with the earlier observed tumor incidences in the cancer tests. The total genotoxic dose that leads to a doubling of the tumor incidences was estimated to be the same in both species, 9 to 10 mmol/L×h EB-equivalents. The study validates the applicability of the multiplicative cancer risk model to genotoxic chemicals. Furthermore, according to this evaluation, different epoxide metabolites are predominating cancer-initiating agents in the cancer tests with BD, the diepoxide in the mouse, and the monoepoxides in the rat. [Cancer Res 2008;68(19):8014–21]

Introduction

1,3-Butadiene (BD) is extensively used in polymer production and is also formed in incomplete combustion of organic matter and is found in, e.g., car exhaust and tobacco smoke (1, 2). Epidemiologic studies have shown an increased leukemia incidence in workers exposed to BD, which underlie the classification of BD by the US Environmental Protection Agency (US EPA) and IARC as a “human carcinogen” (2).³ However, the cancer risk assessment of BD has been encumbered with large uncertainties. The cancer tests in the mouse and the rat with exposures to BD through inhalation showed a large difference in sensitivity to BD between the two species, the mouse being the more sensitive (3–5). Genotoxicity of BD has been comprehensively reviewed (e.g., in refs. 2, 6, 7), and it

is clear that BD induces genotoxic effects in several test systems primarily through its reactive epoxide metabolites 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), and 1,2-epoxy-3,4-butanediol (EBdiol). DEB has been shown (*in vitro*) to be a much more potent inducer of mutations than EB and EBdiol (8, 9). The interspecies difference in the sensitivity to BD in the cancer tests points, on the basis of the genotoxicity tests, toward differences in the internal dose of DEB, due to differences in the rates of formation and detoxification of DEB.

A dose concept for genotoxic electrophiles has been developed where the dose is defined as the concentration over time (area under the concentration curve, AUC; ref. 10). The dose in blood of a reactive metabolite could be calculated from levels of specific adducts to blood proteins (11). Methods for measurement of adducts from EB, DEB, and EBdiol to NH₂-terminal valines in hemoglobin (Hb) have been developed, and data useful for calculations of dose in blood of the epoxide metabolites in BD-exposed mice and rats have been published.

The main objective of this study is to evaluate whether the difference in outcome in cancer tests with BD between the mouse and the rat could be predicted quantitatively on the basis of the doses in blood (AUC) of the epoxide metabolites, EB, DEB, and EBdiol, and their respective relative mutagenic potency. The AUC in the cancer tests are inferred from published (12) Hb adduct levels measured in short-term inhalation studies with BD in rats and mice. To correlate the response in cancer tests to the total genotoxic dose of the epoxides in blood, the relative genotoxic potency (from *in vitro* data) of the respective epoxide has to be considered. The cancer test data are then evaluated on the basis of the total genotoxic dose with the application of a multiplicative cancer risk model.

The multiplicative risk model gives an estimate of the doubling dose (DD) of the causative genotoxic agents, this is the dose that is required to induce a doubling of the tumor frequency. The risk model assumes that the slope of the dose-response is proportional to the background risk, and that the excess relative risk is common for the different responding sites. In addition, it is indicated that the relative risk increment according to this model is approximately independent on species and sex (13). This model has been shown to provide a way of predicting the tumor incidence in long term animal tests with ionizing radiation and also in cancer tests with ethylene oxide and acrylamide (13, 14). The model has been shown to predict the tumor incidence among A-bomb survivors, and the life-time excess relative risk estimates for different solid cancer sites were approximately the same (15, 16). It should be noted that to be able to apply the model to genotoxic agents and

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doi:10.1158/0008-5472.CAN-08-0334

³ International Agency for Research on Cancer (IARC). 1,3-Butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride and vinyl bromide). IARC monographs on the evaluation of carcinogenic risks to humans. Vol. 97, in preparation.

perform interspecies extrapolation, it is a prerequisite to measure the lifetime internal doses (AUC) of the causative agents.

The evaluation of the cancer test data for BD is a challenge because of the large species difference in sensitivity to BD, and because all three epoxide metabolites are genotoxic and could contribute to the initiation of tumors. This study shows the applicability of the multiplicative cancer risk model to the data for BD and support the usefulness of the suggested methodology.

Materials and Methods

Long-term inhalation exposures to BD in cancer tests. Data from two long-term cancer tests with inhalation of BD in Sprague-Dawley rats and B6C3F₁ mice are considered for the evaluation in this study (5, 17). The animals were treated for 6 h per day, 5 d per week in ~2 y. Duration of exposures in the different cancer tests were: 3,090 h (female and male mouse), 3,150 h (female rat), and 3,330 h (male rat). Groups of 50 mice were exposed to 0, 6.25, 20, 62.5, 200, or 625 ppm, and groups of 100 rats were exposed to 0, 1,000, or 8,000 ppm. All exposure doses and all tissues showing a significant response in the cancer tests, as reported in the review of US EPA (2), were used in the evaluation. Table 1 summarizes the target organs and types of neoplastic lesions analyzed.

Short-term inhalation exposure to BD. Published data from short-term inhalation exposures to BD in mice and rats were used for the calculation of doses in blood (AUC) in the cancer tests. From the Hb adduct levels from EB, DEB, and EBdiol measured by Boysen and colleagues (12), we calculated the AUC of the respective epoxide per exposure dose of BD. These data were then used for the calculation of the lifetime doses in blood of the epoxides in the 2-y cancer tests with BD (2). In the short-term studies, Hb adduct levels have been measured in female B6C3F₁ mice exposed to 3, 62.5, or 1,250 ppm for 2 wk (5 d per week, 6 h per day). Furthermore, Hb-adduct data from female F344 rats exposed to 3 or 62.5 ppm for 2 weeks are available. Data from female and male CrI:CD rats (Sprague-Dawley) exposed to 1,000 ppm of BD for 13 wk (12, 18) were also included in the present study.

Dose in blood inferred from levels of Hb adducts. The dose (D) of a genotoxic agent is defined as the concentration (C) over time often called the AUC [in (mol \times L⁻¹ \times h) = (M \times h)] as shown in Eq. 1 (10).

$$D = \int_t C(t)dt \quad (\text{Eq. 1})$$

The dose in blood could be inferred from the measured Hb adduct level [A in (mol \times g⁻¹)], if the rate constant for the reaction is known (in this case, the formation of an adduct to NH₂-terminal valine in Hb; k_{val} in [L \times g⁻¹ \times h⁻¹]). Eq. 2 shows the simple expression for D when the adduct level is measured a short time after acute exposure.

$$D = \frac{A}{k_{val}} \quad (\text{Eq. 2})$$

For the monoepoxy metabolites, EB and EBdiol, adducts with N-termini in Hb were measured as *N*-(2-monohydroxybutenyl)valine (EB-Val) and *N*-(2,3,4-trihydroxybutenyl)valine (EBdiol-Val), respectively. These Hb adducts were measured after detachment as fluorinated derivatives according to the modified Edman method by gas chromatography-tandem mass spectrometry (19, 20). DEB forms a ring-closed adduct, a pyrrolidine, in the reaction with NH₂-terminal valine in Hb [*N,N*-(2,3-dihydroxy-1,4-butadiyl)valine, called DEB-Val; refs. 21–23]. The DEB-Val adduct levels were measured by liquid chromatography-tandem mass spectrometry after enzymatic degradation of Hb and enrichment of the DEB-modified NH₂-terminal α -heptapeptide by immunoaffinity chromatography. For further information on the analytic conditions, see Boysen and colleagues (12). With this method, only the adduct with the α -chain is measured. The reported DEB-Val adduct level is expressed in per gram globin (12). By multiplying the measured DEB-Val adduct level with a factor of 2, the adduct level is here expressed per g α -chain (assuming 50% of the globin weight is α -chain).

The different k_{val} (L·g⁻¹·h⁻¹) for the epoxide metabolites of BD are earlier determined to be as follows: for the mouse, EB 2.9·10⁻⁵ (24), DEB 4.7·10⁻⁵ (21), and EBdiol 2.1·10⁻⁵ (21); and for the rat, EB 4.0·10⁻⁵ (25), DEB 5.5·10⁻⁵ (21), and EBdiol 2.3·10⁻⁵ (21). The k_{val} of DEB refers to the reaction rate with the α -chain N-termini only (21).

For the calculation of the AUC, the published Hb adduct levels of the three epoxide metabolites have been adjusted with regard to the duration of exposure and the disappearance of Hb adducts due to the life span of erythrocytes (t_{er} ; ref. 26). For the strains used in the cancer tests, the t_{er} is

Table 1. Target organs and types of neoplastic lesions with significant response in the mouse and rat cancer test with BD

		Mice			Dose (ppm)				
Sex	Target organ	Neoplastic lesion	Time (h)	0	6.25	20	62.5	200	
Female	All	Malignant lymphoma	3,090	6/50	12/50	11/50	7/50	9/50	
	All	Histiocytic sarcoma		3/50	2/50	7/50	4/50	7/50	
	Heart	Hemangiosarcoma		0/50	0/50	0/50	1/49	21/50	
	Lungs	Adenoma, adenocarcinoma or carcinoma		4/50	15/50	19/50	24/50	25/49	
	Forestomach	Squamous cell papilloma or carcinoma		0/50	0/50	3/50	2/50	4/50	
	Liver	Hepatocellular adenoma or carcinoma		15/49	14/49	15/50	19/50	16/50	
	Harderian gland	Adenoma or carcinoma		8/50	10/50	7/50	15/50	20/50	
	Ovary	Benign or malignant granulosa cell tumor		1/49	0/49	1/48	9/50	8/50	
	Mammary gland	Carcinoma		0/50	2/50	2/50	6/50	11/50	
		Rat			Dose (ppm)				
Sex	Target organ	Neoplastic lesion	Time (h)	0	1000	8000			
Male	Pancreas	Exocrine adenoma	3,330	3/100	1/100	10/100			
	Brain	Glial cell		1/100	4/100	5/100			
	Testis	Leydig cell tumor		0/100	3/100	8/100			
Female	Uterus	Sarcoma	3,150	1/100	4/100	5/100			
	Thyroid gland	Follicular cell adenoma		0/100	2/100	10/100			
	Mammary gland	Benign or malignant		50/100	79/100	81/90			

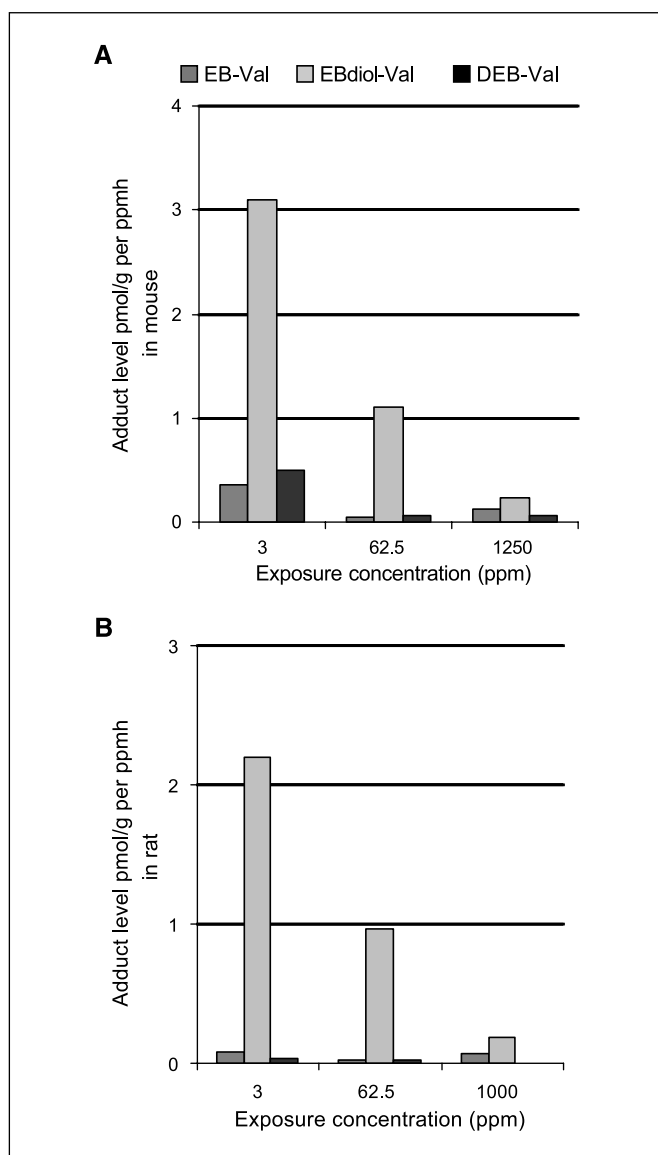


Figure 1. Adduct levels per ppm \times h in (A) the mouse (female) and (B) the rat (female) after inhalation exposure for BD. Published data (12) on Hb-adduct levels adjusted according to Materials and Methods.

~ 40 d in the mouse and ~ 60 d in the rat (27, 28). The correction factors (considering t_{er} and duration of exposure) used are in the range 1.1 to 3.0. The adjusted levels of Hb adducts at the different exposures are summarized in Fig. 1.

Calculations of dose equivalents. The evaluation of the cancer tests of BD according to the multiplicative model was based on the AUC of the genotoxic epoxide metabolites EB, DEB, and EBdiol at the different levels of exposures. Furthermore, the differences in the mutagenic potency of the epoxides were considered to calculate a total genotoxic dose.

In a study using human TK6 cells, it was shown that EB, DEB, and EBdiol were effective at inducing mutations in both the *hprt* and *tk* loci (8). The initial treatment concentrations leading to ~ 2 -fold increases in mutant frequency are listed in Table 2. The rates of disappearance of the epoxides in the medium during treatment will have an influence on AUC of the epoxides (see Eq. 1). AUC was estimated through approximation of the disappearance during the treatment (24 h) with rates of hydrolysis (see Eq. 1; Table 2). Using EB as reference, the relative mutagenic potency per AUC was then estimated with regard to the efficiency of inducing mutation in the *hprt* loci; it was 1:32:0.21 of EB, DEB, and EBdiol,

respectively (Table 2). The *hprt* loci was chosen because the availability of *in vivo* data for possible comparison. The absolute values of the treatment doses of the epoxides are encumbered with uncertainties; however, the comparison of the mutagenic potency per AUC gives a better estimate than the conventional approach using comparison of mutagenic potency per initial treatment concentration.

The relative genotoxic potency could then be used to convert the AUC of EB, DEB, and EBdiol to a total genotoxic dose by multiplying the dose D (AUC) of the respective epoxide with the corresponding mutagenic potency (1:32:0.21; Eq. 3; Table 3). This is expressed as EB dose-equivalents (mmol/L \times h) per exposure dose of BD (ppm \times h).

$$EB \text{ dose-equivalent} = D_{EB\text{-equi.}} = D_{EB} \times 1 + D_{DEB} \times 32 + D_{EBdiol} \times 0.21 \quad (\text{Eq. 3})$$

EB dose-equivalents in cancer tests. The total genotoxic doses from the epoxide metabolites of BD in the cancer tests with mice and rats were calculated on the basis of EB-equivalent dose from the inhalation exposures (Table 3). For the rat cancer test, the total genotoxic dose was inferred from EB-equivalent dose in both sexes at the exposure level of 1,000 ppm. No Hb-adduct measurements are available above 1,000 ppm in the rat. The highest exposure at 8,000 ppm in the rat cancer test will therefore be discussed separately, see "High exposure levels" in the Discussion section.

The total genotoxic doses in the mouse cancer test were estimated from the EB-equivalent doses in blood obtained at the exposure levels of 3, 62.5, and 1,250 ppm. Different scenarios were applied for the calculation of the AUC at the exposure levels in the cancer test (6.25, 20, 62.5, and 200 ppm; 625 ppm excluded due to high mortality). The primary scenario assumes a linear increase up to 6.25 ppm from the measurement at 3 ppm. The estimate for 20 ppm was then obtained by linear extrapolation between the predicted value at 6.25 ppm and the observed value at 62.5 ppm. The alternative AUC for 6.25 and 20 ppm were based on direct interpolation between the measurements at 3 and 62.5 ppm. These two scenarios are illustrated in Fig. 2 as EB-equivalent doses. Two scenarios were also applied for estimating the EB-equivalent dose at the highest exposure level at 200 ppm. This will be further discussed below (see "High exposure levels" in the Discussion).

Cancer risk model. The model known as the excess relative risk model (or multiplicative model), often applied in radiation research, was fitted to the tumor incidence data. This model implies that the fraction of animals with tumors in target tissue i , i.e., $P_i(D)$, can be described by the following function:

$$P_i(D) = 1 - e^{-P_i^0(1+\beta D)} \quad (\text{Eq. 4})$$

D denotes the dose (AUC) in target tissues of the genotoxic agent. In the present evaluation of cancer tests with BD, the dose denotes the total dose in blood of cancer-initiating agents (expressed as EB dose-equivalents). For specific organs, the dose in blood has to be related to the target dose (see below).

P_i^0 represents the cumulative hazard for tumor formation in the target tissue i among unexposed animals, and β is the common relative excess risk per unit of dose. We express the relative potency as the DD, which is the same as $1/\beta$, that is the dose yielding a cumulative hazard of $2P_i^0$. When we refer to a model with a quadratic term, the following model is fitted:

$$P_i(D) = 1 - e^{-P_i^0(1+\beta D+\beta D^2)} \quad (\text{Eq. 5})$$

The variables of these nonlinear functions were estimated by binomial regression models. The 95% confidence intervals (95% CI) for the estimated DDs were obtained by the profile likelihood method. The fit of the model is displayed as observed versus predicted tumor incidence (Fig. 3).

Results

Doses in Blood of Epoxide Metabolites of BD

Hb adduct levels. Methods for Hb adduct measurements were not available at the time for the cancer tests with BD, and AUC in

Table 2. Relative mutagenic potencies of BD epoxide metabolites. Relative potency per AUC (concentration over time, $\mu\text{mol/L} \times \text{h}$) estimated from initial treatment concentrations inducing a doubling of the mutation frequency in TK6 cells *hprt loci* (and *tk loci*; ref. 8)

	EB	DEB	EBdiol
Initial exposure concentration ($\mu\text{mol/L}$)	150 (100)	3.5 (1)	450 (350)
AUC ($\mu\text{mol/L} \times \text{h}$)	2.1 (1.4)*	0.066 (0.019) [†]	10 (7.8) [‡]
Relative mutagenic potency per $\mu\text{mol/L} \times \text{h}$	1	32 (74)	0.21 (0.18)

*Hydrolysis rate (half life), 0.05 h^{-1} (14 h) (49).

[†]Hydrolysis rate (half life), 0.021 h^{-1} (34 h) (H. Motwani, C. Fred, J. Haglund, B.T. Golding, and M. Törnqvist. Cob(I)alamin as an analytical tool to study 1,3-butadiene metabolism in human, mouse and rat S9 liver fractions. Poster presentation at the Gordon Research Conference on Vitamin B12 & Corphins, Biddeford, Maine, July 1 to 6, 2007. Manuscripts in preparation).

[‡]Hydrolysis rate (half life), 0.0062 h^{-1} (112 h) (H. Motwani et al., in preparation).

blood had to be obtained from later, similar short-term exposure experiments with mice and rats. Assuming that the metabolic rates are about the same irrespectively if the exposure continues for 2, 13, or ~ 100 weeks, the published Hb adduct measurements from inhalation experiments with BD were used for estimation of doses in blood of the epoxides of BD over the lifetime in the cancer tests. These were then used to calculate the total genotoxic dose, expressed as EB-equivalent dose, considering the genotoxic potency of the individual epoxide metabolites.

Results from the Hb adduct measurements of EB, DEB, or EBdiol in the mouse and the rat in exposure studies with BD have earlier been compared and used for evaluation of the species differences in intermediate steps in the metabolism of BD (29). The data on adduct levels of EB-Val and EBdiol-Val in Hb from mice and rats in the study of Boysen and colleagues (12) were in agreement with the earlier studies in rodents on Hb-adduct levels after treatment with BD (20, 25, 30). The study of Boysen and colleagues (12), however, is the first to report data on DEB-Val adduct levels after treatment with BD, and the Hb-adduct data from this study was therefore used for the calculations in the present study.

A comparison between the two species of the observed Hb-adduct levels after 2 weeks of exposure, without the adjustments

for the disappearance rate of Hb adducts, gives qualitatively similar result as the comparison with regard to the AUC as calculated in the present study (see below). However, the AUC of the epoxides inferred from the adducts levels are necessary for an accurate quantitative comparison between species and allow the calculation of total genotoxic doses, as EB dose-equivalents.

Calculated AUC of epoxide metabolites in short-term inhalation exposures with BD. Generally, the AUC of the epoxide metabolites increased with exposure in both the mouse and the rat. For all three epoxides, the highest AUC per exposure dose were observed at 3 ppm (Table 3). This indicates a more effective metabolism to epoxides at low exposure levels. The AUC of EBdiol increased with exposure dose, however, not linearly, probably due to saturation of metabolizing enzymes. This has also been observed through measurements of Hb adducts in earlier studies with exposure to BD or to its epoxide metabolites (20, 30), and recently through measurement of steady-state concentrations of epoxide metabolites in blood after a single exposure to BD (31). A predominant difference between the mouse and the rat was observed with regard to the AUC of DEB, which at 3 ppm already was high in the mouse, and ~ 16 times lower in the rat. The lower AUC of DEB per exposure dose at 62.5 ppm in the rat indicates an

Table 3. The doses in blood of EB, DEB, and EBdiol per treatment dose of BD ($\text{mmol/L} \times \text{h/ppmh}$) in the mouse and the rat are based on data from short-term inhalation exposures for 6 h per day, 5 d per week

Duration	Exposure (ppm)	Doses in blood ($10^{-6} \text{ mmol/L} \times \text{h/ppmh}$)				
		EB	DEB	EBdiol	$D_{\text{EB-equi}}$	
Mice (B6C3F1) 2 wk (female)	3	12	14	150	500	
	62.5	1.6	1.8	53	71	
	1,250	4.0	1.7	11	62	
Rats (F344) 2 wk (female)	3	2.1	0.90	93	50	
	62.5	0.66	0.42	42	23	
Rats (CrL:CD) 13 wk	Female	1,000	1.7	0.016	8.0	3.9
	Male	1,000	1.1	0.0047	4.0	2.1

NOTE: The doses in blood are summarized to a total genotoxic dose ($D_{\text{EB-equi}}$; see Eq. 3 in Materials and Methods).

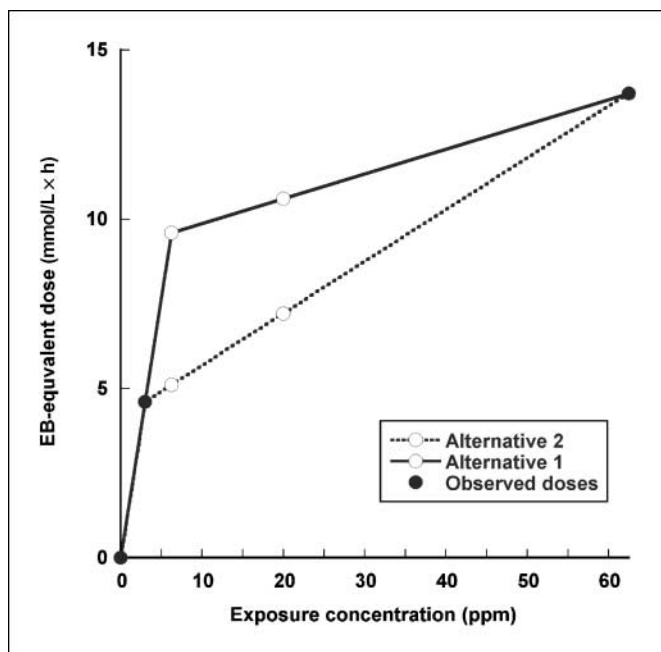


Figure 2. The relationship between exposure concentration (0–62.5 ppm) and the EB-equivalent dose for the mouse (female), with two alternative interpolation scenarios.

almost saturated metabolism. This is a major cause of the difference in the calculated EB dose-equivalent for the two species.

Calculated AUC in the mouse and the rat in the cancer tests. The exposure levels in the cancer tests considered were as follows: the mouse, 0 to 62.5 ppm; the rat, 0 to 1,000 ppm. At the high exposure levels (200 and 625 ppm in the mouse and 8,000 ppm in the rat), saturation of the metabolism and also toxic effects are more pronounced. This is further discussed below (see “High exposure levels” in the Discussion). The total genotoxic dose (EB-equivalents) in the mouse increases with the BD exposure levels 0 to 62.5 ppm (Fig. 2). The EB-equivalent dose in the cancer test with rats exposed to 1,000 ppm was estimated to 12.2 mmol/L × h in females and to 6.8 mmol/L × h in males.

DD in Cancer Tests

The relative excess risk per unit of dose (β in Eq. 4 and 5) may be expressed as $DD = 1/\beta$ common for all tumor sites. In this study, the dose in blood (AUC) of the epoxide metabolites is considered as a basis for the DD. The DD was estimated as the lifetime EB dose-equivalent (mmol/L × h) for all sites (Table 4).

In female mice, the DD of the EB-equivalent dose was estimated to 10.0 and 8.5 mmol/L × h in the two scenarios of AUC (Fig. 2; Table 4). If the dosimetry for female mouse was applied also to the cancer test of male mouse, for which no dosimetry data are available, a similar DD was obtained (17.9 mmol/L × h). For rats (female and male) the common DD was 8.9 mmol/L × h. The multiplicative model based on doses in blood yields an adequate fit for the cancer test data with a good prediction of observed number of tumors (Fig. 3).

The DD is thus very similar, ~9 mmol/L × h, irrespectively of species or sex according to this evaluation (Fig. 3; Table 4). Quantitative differences in metabolism between the two species (Table 3) results in that DEB is the major contributor (close to 90%) to the total genotoxic dose in the mouse, whereas in the rat, DEB represents only a minor fraction (5–10%) of the total genotoxic dose (Eq. 3; Table 3). Therefore, it may be concluded that DEB is the

predominating cause of cancer initiation in the mouse, and EB together with EBdiol (40–50% contribution of each) are the causative agents in the rat.

Discussion

Cancer risk estimates for target sites. To estimate a common risk coefficient, β , for all tumor sites on the basis of dose in blood, it is a necessity that the cancer causative agent(s) is (are) evenly distributed in the different organs. Measurements of the concentrations of EB and DEB in mice and rats after a 4-hour exposure to 62.5 ppm BD in earlier studies have shown that the concentration of the respective epoxide is similar in all vessel-rich organs (32). These results are strengthened by reports on the DNA adducts from EB and EBdiol, showing that approximately the same level of the respective adduct was present in all tissues examined (33, 34). However, the concentrations of the epoxide metabolites in organs were found to be one half of the concentration in blood (32). This has earlier also been shown for the chemically related compound ethylene oxide through the comparison of Hb- and DNA-adduct levels (35). This supports that the DD, estimated from the dose in blood, should be reduced by 50% to give an appropriate estimate of the dose in target tissues in the cancer tests with BD (13, 36).

High exposure levels. In the primary analyses of the published cancer test data, the exposure level 8,000 ppm for the rat and 200 and 625 ppm for the mouse were not included because the dosimetry data were uncertain. Furthermore, the survival was heavily affected at 625 ppm in the mouse where ~90% of the animals were dead already after 60 days and these data were therefore not considered.

When including the highest exposure level, 8,000 ppm, for the rat, and assuming a proportional increase of the AUC of the epoxides from 1,000 ppm, a highly significant quadratic dose

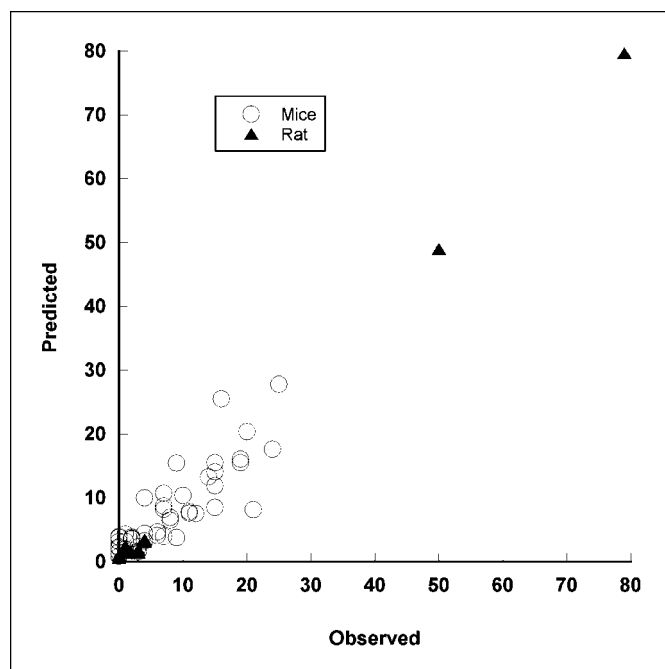


Figure 3. Observed and predicted incidence of neoplasms in mice and rats applying the linear excess relative risk model. The model using the doses 0 to 62.5 ppm (primary scenario) for mice and 0 to 1,000 ppm for rats is shown. A residual analysis shows no indications of lack of fit.

Table 4. Estimated DDs of EB-equivalents in mice and rats exposed to BD

Species	Doses (ppm)	Model	Dose scenario	DD (mmol/L × h; 95% CI)
Mice ^{*,†} (female)	0–62.5 ppm	Linear	1	10.0 (5.5–21.1)
Mice (female)	0–62.5 ppm	Linear	2	8.5 (4.8–17.1)
Rat (female, male)	0–1000 ppm	Linear	—	8.9 (5.1–18.4)

*Male mice 0 to 62.5 ppm (AUC approximated as dose alt 1 for female): DD = 17.9 (9.4–49.3) mmol/L × h.

†Female mice 0 to 62.5 ppm excluding neoplasms of the liver (AUC approximated as dose alt 1): DD = 6.3 (3.2–13.2) mmol/L × h.

component ($P < 0.001$) was needed to provide an adequate fit. A secondary scenario assuming complete saturation, i.e., the AUC of the epoxides at 8,000 ppm is the same as at 1,000 ppm, was tested. This is supported by measurements of epoxide metabolite concentration after single exposures to BD, which showed that formation of DEB and EBdiol will be saturated (31, 37) and that only the EB concentration will continue to increase at exposure above 1,000 ppm (37). With this assumption, a linear dose-response model provided an adequate fit and the DD estimate of EB-equivalents [DD = 7.2 (4.5–12.5) mmol/L × h] was essentially unchanged compared with the analysis restricted to 1,000 ppm.

When including the cancer data at 200 ppm in mice, we considered two scenarios to estimate the AUC at this exposure level: either applying linear interpolation (between 62.5 and 1,200 ppm), or assuming proportionality to the AUC at 62.5 ppm. In both cases, a linear model yielded an estimated DD of ~15 mmol/L × h. However, when testing curvature of the dose-response, a negative quadratic dose-term was found, which is most probably due to an increased fraction of animals dying at 200 ppm before developing tumors. In the first case, this term was not statistically significant and, in the second case, it was ($P < 0.05$).

Cancer risk model. The risk model used implies that the risk increment per unit dose of the genotoxic agent in different responding tissues is proportional to the background incidence in the respective tissue. The interpretation is thus that the background promotive conditions determine the spectrum of spontaneous tumors as well as tumors induced by an exogenous genotoxic agent. At very high doses of exposure in cancer tests, the exposure might influence the promoting conditions, which should be reflected in the quadratic term in the risk model (Eq. 5). This is described more in detail in refs. 13, 38.

This is a rather crude, mechanism-based model for cancer risk assessment of genotoxic agents, which is manageable and straightforward when dosimetry data are available. The model seems not to be suitable for assessment of nongenotoxic carcinogens, which are likely to be tissue specific and probably also much more species specific. However, if certain tissues show deviations from the multiplicative model, it may indicate that the substance has other modes of action than genotoxic.

There are potential biases in our estimated DDs. The inclusion of all sites showing a significant increase in tumor incidence after exposure may lead to an overestimation of the relative potency because strong responses due to chance may be overrepresented. This problem would be particularly pronounced in the case of studies with low statistical power, which can be due to small sample sizes, low potency of the chemical tested and/or inadequate dosing. The second source of bias is related to the fact that the exposure decreases the overall survival. Because we used the crude

tumor rates in our analyses the excess mortality due to early onset of lethal tumors or death due to toxicity may be a competing risk, leading to an underestimation of the risk. However, exposure levels up to 62.5 ppm for the mouse and 1,000 ppm for the rat did not substantially effect the overall survival.

Relative genotoxic potency. In the present study, the relative *in vitro* mutagenic potencies of the three epoxide metabolites of BD were used to calculate the EB-equivalent doses from the doses in blood of the epoxides. The EB-equivalent doses were then used as a measure of the total genotoxic doses in the cancer tests (see “Calculations of dose equivalents” above). The reliability of the estimated relative genotoxic potencies is supported by earlier results from a similar study with ethylene oxide. Approximately the same DD in blood (~11 mmol/L × h) was observed for ethylene oxide in cancer tests with the mouse and the rat, as was obtained for the EB-equivalent (~9 mmol/L × h) in this study (13). *In vitro* mutation data support that the genotoxic potency (per AUC) of an EB-equivalent and of ethylene oxide is about the same (8, 39, 40). This means that we expect approximately the same DD for ethylene oxide and the EB-equivalent.

There are some *in vivo* mutation data with parallel Hb adduct measurement for the BD epoxide metabolites, but the data are not sufficient for estimating the *in vivo* relative genotoxic potency. However, the observed mutation frequency of BD and 3-butene-1,2-diol (precursor of EBdiol) *in vivo* (41, 42) is by and large in agreement with the one estimated (data not shown) from AUC of the epoxides and *in vitro* relative genotoxic potency obtained in the present study.

Comparison with epidemiologic studies. Workers exposed to high concentrations of BD have shown an increased mortality in leukemia (reviewed in ref. 2). However, the most comprehensive studies of low BD exposure were negative with regard to *HPRT* gene mutation and cytogenetic response, although measured Hb adduct levels showed a significant increase with exposure (43, 44). In these studies, neither the mutational nor the cytogenetic responses showed any association with genotypes. There are, however, other studies in humans showing a significant difference in levels of mutations and cytogenetic effects related to BD exposures (e.g., refs. 45, 46).

The large species difference in sensitivity to BD observed in the rodent cancer tests are according to the evaluation due to a difference in the metabolism to DEB. *In vitro* metabolism data and Hb adduct levels indicate that humans generate low doses of DEB and, as for rat, high exposure levels are most probably required to reach a sufficient *in vivo* dose of the epoxides to cause a detectable genotoxic/carcinogenic response. A causal relationship between leukemia and high cumulative exposure and high intensity of exposure to BD is supported by a study using an excess relative risk model, similar to the model used in the present evaluation (47). The DD estimated from the linear regression was 595 ppm-year.

When corrections for peak exposures were performed, the DD was estimated to ~5,300 ppm-year. The DD observed in the rat in the present evaluation (8.9 mmol/L × h EB-equivalent; Table 4) corresponds to ~1,400 ppm-year (700 ppm for 2 years).

An uncertainty with regard to the extrapolation to humans from toxicologic data in general concerns the dose-response relationship at very low doses. AUC measurement in blood, e.g., by Hb adduct measurements, bypass the problems of species- and high-to-low dose differences in metabolism of reactive compounds/intermediates. Deviations from linearity in dose-response due to DNA repair, etc., at very low exposure levels could exist for certain compounds (48). These are, however, not detected with the sensitivity in the standard test systems. The comparison above of data for the rat with the epidemiologic data from occupational exposure to BD indicates no obvious disagreement in estimated risks.

Conclusion

Published cancer tests with BD in mice and rats have been evaluated with regard to the applicability of a multiplicative (relative) risk model to the tumor frequency data and on the basis of total genotoxic doses in blood. The good fit of the data to the multiplicative model shows that the dose-response is proportional to the background risk, i.e., the background variations in promotive conditions in the different tissues influence the outcome in tumor development. According to this evaluation, the relative risk coefficient obtained is approximately independent of species, sex, and tumor site. Although the data from rats is relatively weak and

we also have some potential sources of bias, the apparently very large difference in carcinogenic potency of BD between the species can be quantitatively explained by the suggested approach. Furthermore, it is notable that different metabolites of BD are found to be the cancer causative agents in mice and rats, which underline a weakness of cancer tests as a basis for risk estimation and extrapolation of risks to humans.

Comparisons of mutagenic potencies (*in vitro* and *in vivo*) together with internal dose measurement of the potential causative carcinogenic agent(s) would, in the opinion of the authors, reduce the need for long term cancer tests of genotoxic agents. In conclusion, the results from the present study further validate the applicability of the multiplicative cancer risk model to genotoxic chemicals as an improved basis for cancer risk estimation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/28/2008; revised 7/23/2008; accepted 7/25/2008.

Grant support: The Swedish Animal Welfare Agency, the Swedish Cancer and Allergy Fund, the Swedish Energy Agency, and the Swedish Research Council Formas.

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We thank the late Prof. Lars Ehrenberg who was the initiator of this approach and contributed to this work as a great inspirer and as an inexhaustible source of knowledge, and Assoc. Prof. Siv Osterman-Golkar for critical reviewing of the manuscript.

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