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The use of non-tumor data in cancer risk assessment: reflections on butadiene, vinyl chloride, and benzene☆

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Abstract

The estimation and characterization of a cancer risk is grounded in the observation of tumors in humans and/or experimental animals. Increasingly, however, other kinds of data (non-tumor data) are finding application in cancer risk assessment. Metabolism and kinetics, adduct formation, genetic damage, mode of action, and biomarkers of exposure, susceptibility, and effects are examples. While these and other parameters have been studied for many important chemicals over the past 30–40 years, their use in risk assessments is more recent, and new insights and opportunities are continuing to unfold. To provide some perspective on this field, the ILSI Risk Science Institute asked a select working group to characterize the pertinent non-tumor data available for 1,3-butadiene, benzene, and vinyl chloride and to comment on the utility of these data in characterizing cancer risks. This paper presents the findings of that working group and concludes with 15 simple principles for the use of non-tumor data in cancer risk assessment.

Keywords: Cancer; Risk assessment; Biomarkers; 1,3-Butadiene; Benzene; Vinyl chloride; Principles

1. Introduction

Quantitative estimation of human cancer risk from exposure to chemicals has traditionally been based almost exclusively on tumor incidence in experimental animals. While improvements in carcinogenicity testing in experimental animals (Milman and Weisburger, 1994; NTP, 2002) and structure–activity correlations have provided new insights for the prediction of carcinogenic potential, the induction of tumors remains the central focus of efforts to identify and regulate chemical carcinogens (IARC, 2000; U.S. EPA, 1996; NTP, 2001). It is widely recognized that reliance on animal tumor

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incidence data, with its inherent need to extrapolate to humans, introduces considerable uncertainty into the risk assessment process and that this process needs to be expanded to include other types of data that might reduce uncertainty.

There is a growing recognition that non-tumor data can play an important part in cancer risk assessment (U.S. EPA, 1996; NTP, 2001, 2002; Olin et al., 1995). Such data include information on metabolism, formation of DNA adducts, various types of genetic damage, pharmacokinetic models, information on mode of action, etc. However, exactly how these data can be used to improve risk estimates is less apparent and, in fact, may be case-dependent. To examine this issue, the ILSI Risk Science Institute convened a working group to characterize the types of non-tumor data available for three well-known human carcinogens: 1,3-butadiene (BD), vinyl chloride (VC), and benzene (BZ) and to suggest how these data might be used in cancer risk assessment. This paper summarizes the observations and conclusions of this working group. This contribution begins with a discussion of possible uses of non-tumor data, followed by an overview of non-tumor data for each of the three example carcinogens, and concludes with a statement of general principles for the appropriate use of non-tumor data in cancer risk assessment.

2. Non-tumor data

A major potential use of non-tumor data in cancer risk assessment is to establish the mode of action by which toxicity is induced, starting with metabolism and interaction of the active agent with cellular components and proceeding through biological changes resulting in tumor formation. The use of animal bioassay data to identify human carcinogens assumes that the qualitative response (mode of action) in animals and humans is similar. Confidence in the use of animal data in risk assessment is increased when the mode of action is demonstrated to be the same in humans and animals.

Understanding the mode of action allows increased confidence in extrapolation across species and from high to low doses. To demonstrate a particular mode of action, it is necessary to hypothesize the sequence of events leading to the outcome of interest (i.e., cancer), obtain experimental verification of the key steps, and summarize the information in a way that allows the validity of the hypothesis to be assessed (Sonich-Mullin et al., 2001).

Use of a mode of action approach recognizes that, although all of the details may not be known, it is possible to move forward using a hypothesized mode of action supported by data. In its recent reevaluation of dioxin, the U.S. Environmental Protection Agency (EPA) relied primarily on mode of action evidence together with strong animal cancer data and limited human evidence to conclude that dioxin is a human carcinogen. Knowledge of mode of action can also be used to disregard animal cancer data when it is determined that humans are not affected by the same mode of action, as was the case for α_{2u}-globulin-associated kidney tumors, urinary bladder tumors induced by calculi, and thyroid follicular cell tumors resulting from perturbation of thyroid hormone homeostasis in rats (U.S. EPA, 1991; U.S. EPA, 1998; Capen et al., 1999).

Beyond their use in the qualitative hazard characterization process, non-tumor data may play an important role in other aspects of cancer risk assessment. In the introduction to the Dose-Response Assessment section of its Proposed Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1999), EPA discusses the use of non-tumor response data to enhance dose–response analysis. EPA states "response data include measures of key events considered integral to the carcinogenic process, in addition to tumor incidence. These responses may include changes in DNA, chromosomes, or other key macromolecules; effects on growth signal transduction, including induction of hormonal changes; or physiological or toxic effects that affect cell proliferation. Key events are precursors to cancer pathology; they may include proliferative events diagnosed as precancerous, but not pathology that is judged to be cancer. Analysis of such responses may be done along with those of tumor incidence to enhance the tumor dose–response analysis."

The relationship between dose and tumor response observed in a carcinogenesis bioassay study is often the starting point for estimation of the human cancer risk for a chemical, but ancillary non-tumor data are invariably required. Applications of such data include adjustment of dose for interspecies differences in body mass; metabolic rates and pathways; and uptake, distribution and excretion. In some cases, physiologically based pharmacokinetic (PBPK) models have been developed to provide a better measure of dose at the target tissue. Also, ancillary data may be needed for route-to-route extrapolation if the route used in the carcinogenicity study is not the exposure route of concern for humans. PBPK models can be used to describe the uptake and distribution of environmental toxicants in humans and animals and potentially have the ability to organize non-tumor pharmacokinetic data in a manner useful in cancer risk assessment. A range of such models, based on increasingly sophisticated knowledge, allow progressively more accurate characterizations of the dose–response relationship. Despite a desire to construct detailed biologically based models to reduce uncertainty in characterizing risk, EPA has actually used few such models in the regulatory process. Vinyl chloride is an exception.

Cancer risk assessment often requires extrapolation of the dose–response relationship below the dose range of the tumor incidence data. Qualitative and quantitative data for key events involved in the tumor formation

process or for factors (e.g., cell proliferation) that may affect the tumor incidence would be helpful. Data on non-tumor (i.e., precursor) effects might be combined with the tumor data to extend the dose–response below dose levels in animal studies associated with tumor induction. Thus, in some circumstances, surrogates (biomarkers) for dose or dose-related effects that are measures of key events involved in the carcinogenesis process might be used to meaningfully extend the dose–response curve to lower dose levels.

3. Case studies

The purpose of this section is to present three case studies that illustrate the types of non-tumor data that can arise during the study of the carcinogenicity of environmental chemicals. The three case studies lay the basis for the Principles that will be presented in Section 4.

3.1. Butadiene

Butadiene (BD) is a highly volatile four-carbon chemical (C_4H_6; CAS RN106-99-0) made from the processing of petroleum. It is a colorless gas with a mild gasoline-like odor. Worldwide, its annual production is approximately 12 billion pounds, with 3 billion pounds produced in the U.S., making it the 36th highest volume chemical produced in the United States. About 75% of the manufactured 1,3-butadiene is used to make synthetic rubber. In 1985, the U.S. EPA classified BD as a "probable human carcinogen," with a possible upgrade to "known human carcinogen" pending. [*Note added in proof*. In November 2002 EPA revised its assessment of butadiene, to a weight-of-evidence characterization of "carcinogenic to humans by inhalation" (U.S. EPA, 2002).] The International Agency for Cancer Research (IARC) designated BD as a "probable human carcinogen" (Group 2A) in 1992 and again in 1999. Many agencies in many countries have classified BD as a carcinogen or potential carcinogen in humans.

3.1.1. Cancer studies

Three BD cancer bioassays in mice have been conducted. In the first bioassay, male and female mice were exposed to BD at 625 or 1250 ppm (1381.3 or 2762.5 mg/m^3); the study was terminated early because of excess mortality due to lymphomas (Huff et al., 1985). In the second study, female mice exposed to 6.25 ppm (13.8 mg/m^3) showed a significantly increased incidence of lung tumors, and male and female mice exposed to 20, 62.5, 200, or 625 ppm (44.2, 138.1, 442.0, or 1381.3 mg/m^3) showed significantly increased incidences of tumors at multiple organ sites at all exposure levels (NTP, 1993; Melnick et al., 1990). The third bioassay compared tumor incidences in B6C3F1 and NIH Swiss mice, demonstrating that BD-induced lymphomas were not solely attributable to retroviral activation (Irons et al., 1989). The single cancer bioassay in Sprague–Dawley rats exposed to 1000 or 8000 ppm (2210 or 17680 mg/m^3) BD also showed different neoplasms at multiple organ sites (Owen et al., 1987). However, the rats were two to three orders of magnitude less sensitive to cancer induction by BD than the mice. Differential sensitivity between species has been a major source of uncertainty in extrapolating animal data to humans.

Epidemiologic studies have been reported both for workers having exposures to relatively pure BD (e.g., in BD monomer production), and for workers with more complex exposures [e.g., those engaged in BD polymerization such as in the styrene–butadiene rubber (SBR) industry]. The largest study of monomer production workers involved 2795 persons employed at least six months between 1952 and 1994 (Divine et al., 1993; Divine and Hartman, 1996). There were also two smaller studies of monomer production workers (Downs et al., 1987; Ward et al., 1994, 1996a; Cowles et al., 1994). Although increases in hematological malignancies, mainly lymphomas, were reported in one of the small studies and in earlier updates of the large cohort study, such findings were inconsistent. Overall the evidence for cancer induction in monomer production workers has been weak and without dose-related responses. Positive associations between exposures to BD in monomer production workers and malignancies were only observed in workers employed before 1950. The findings were different for SBR workers. The largest SBR industry study of 15,649 men from eight North American SBR plants found an increased leukemia risk (Delzell et al., 1996; Meinhardt et al., 1982; Matanoski et al., 1993; Santos-Burgoa et al., 1992; Macaluso et al., 1997). However, there were no increases in other lymphohematopoietic malignancies. The excess leukemia deaths were in job categories with relatively high BD exposures. Retrospective dose reconstructions suggested an exposure–response relationship for BD, but no relationship to styrene (Macaluso et al., 1997). Overall, the epidemiological findings in BD exposed workers suggest but do not prove carcinogenicity for humans.

3.1.2. BD metabolism

BD is initially oxidized to the 1,2-epoxy-3-butene (EB), a reaction mediated primarily by P450 CYP 2E1 (Csanády et al., 1992; Duescher and Elfarra, 1994) (Fig. 1). Further oxidation of EB produces the 1,2:3,4-diepoxybutane (DEB) (Seaton et al., 1995). Detoxification of EB proceeds by conjugation, mediated by glutathione-*S*-transferase (GST), or by hydrolysis, mediated by epoxide hydrolase (EH). Hydrolysis produces the 1,2-dihydroxy-3-butene (BD-diol) metabolite. Both DEB and BD-diol undergo further conversions in vivo, the former by EH mediated hydrolysis and the

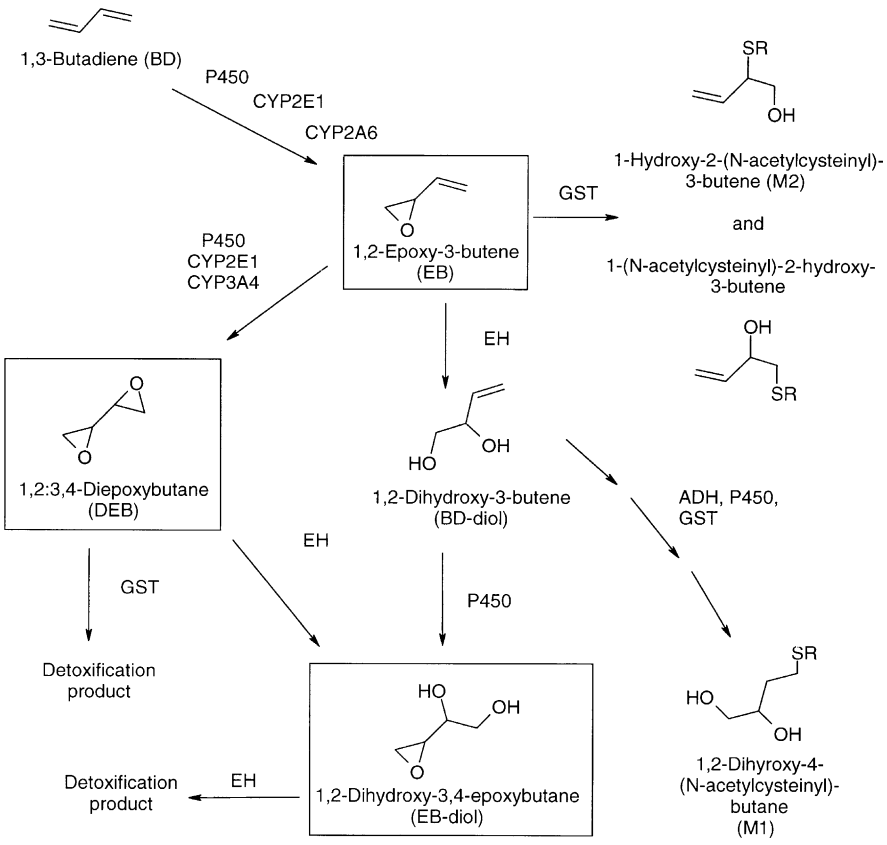


Fig. 1. Partial metabolic scheme for 1,3-butadiene (BD).

latter by P450 mediated oxidation, to produce the 1,2-dihydroxy-3,4-epoxybutane metabolite, known also as butadiene diol-epoxide (EB-diol) (reviewed in Himmelstein et al., 1997). BD-diol can also be metabolized by P450 to hydroxymethylvinylketone (HMVK) (Kemper et al., 1998), which may form $1N^2$-propanodeoxyguanosine DNA adducts in vitro. (Powley et al., 2003). EB, DEB, EB-diol, and HMVK are reactive electrophilic compounds with the potential to form carcinogenic intermediates of BD metabolism in vivo.

Direct GST mediated conjugation of EB with glutathione (GSH) leads to two detoxification products. One of these (i.e., 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene, also known as the urinary M2 compound), as an isomeric mixture with 1-(*N*-acetylcysteinyl)-2-hydroxy-3-butene, is a biomarker of the conjugation detoxification pathway. GST mediated conjugation of HMVK with GSH leads to the production of 1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane (also known as the urinary M1 compound). M1 is a biomarker of the hydrolytic pathway because this detoxification pathway for EB is mediated initially by EH. The ratio M1/(M1 + M2) in urine defines the relative importance of hydrolysis vs. conjugation in detoxification of EB (Bechtold et al., 1994; reviewed in Henderson et al., 1996).

In vitro studies have shown that mice are 2- and 10-fold more efficient than rats in oxidizing BD to EB (Schmidt and Loeser, 1985; Csanády et al., 1992). Furthermore, the second oxidation step to DEB could be mediated in vitro only by mouse liver microsomes (Csanády et al., 1992). In vivo studies of BD metabolism in mice and rats have also shown large inter-species differences. M1/(M1 + M2) ratios in urine for mice and rats exposed to BD by inhalation indicate that conjugation detoxification predominates in mice but that hydrolysis is more important in rats (Henderson et al., 1996).

In summary, mice are more efficient in oxidation of BD to electrophilic metabolites (especially to DEB), while rats are more efficient in hydrolytic detoxification. Chemicals that might be similar to BD in requiring in vivo oxidation to form reactive intermediates are ethylene, propylene, isoprene, and chloroprene.

3.1.3. Molecular dosimetry: hemoglobin and DNA

N-(2-Hydroxy-3-butenyl)valine (HBVal) hemoglobin adducts were produced by EB in both B6C3F1 mice and Sprague–Dawley rats exposed to BD by inhalation, with linearity in the mouse and non-linearity (supra-linearity) in the rat (i.e., at 10–100 ppm BD, mice showed a 5-fold greater adduct concentration than rats while at <10 ppm exposures there was only a 1.7-fold higher concentration in mice) (Osterman-Golkar et al., 1991, 1993). In another study, mice exposed to higher levels of BD showed 2.3- and 1.9-fold greater HBVal adduct concentrations

than did rats for males and females, respectively (Swenberg et al., 2000). The *N*-(2,3,4-trihydroxybutyl)valine (THBVal) hemoglobin adducts are potentially produced by either DEB or EB-diol, although current evidence indicates that they derive almost entirely from the latter (Pérez et al., 1997; Koivisto et al., 1999). After exposure to 1000 ppm (2210.0 mg/m^3) BD by inhalation, the average male and female concentrations of THBVal adducts were 4.3-fold greater in mice than in rats (Swenberg et al., 2000). Within a species, the ratio of THBVal to total HBVal adduct concentrations was 6.3 for male mice, 3.4 for female mice, 1.8 for male rats, and 2.5 for female rats. These data indicate that the EB-diol metabolite is the most abundant electrophilic product of in vivo BD metabolism in both species, with more produced in mice than in rats (Swenberg et al., 2000; Pérez et al., 1997; Koivisto et al., 1999; Koc et al., 1999).

BD metabolites also form covalent adducts with DNA in multiple species (Citti et al., 1984; Tretyakova et al., 1997; Koc et al., 1999; Koivisto et al., 1999). N7-(2-hydroxy-3-butenyl) guanine (HBG) and/or N6 (HB) adenine adducts derived from EB have been found in lung, liver and testes of mice and in lung and liver of rats exposed to BD by inhalation (Koivisto et al., 1996, 1997, 1998; Blair et al., 2000). N7-(2,3,4-trihydroxybutyl) guanine (THBG) adducts have also been detected in liver DNA from mice and rats exposed to BD by inhalation (Koc et al., 1999; Blair et al., 2000; Tretyakova et al., 1998). The exposure response for THBG was markedly supralinear in rats, plateauing at 62.5 ppm, while mice show a lesser slope starting at 62.5 ppm, but continuing to rise to 625 ppm (Koc et al., 1999). In contrast, HBG was linear in both rats and mice. Thus, molecular dosimetry demonstrated that although rats appeared to be 2–3 orders of magnitude less sensitive than mice, some of this difference may have been due to not testing at lower exposures.

In summary, the hemoglobin and DNA adduct data are consistent with the results of metabolic studies in mice and rats, indicating that the former species is more efficient in oxidizing BD to its electrophilic intermediates.

3.1.4. Genotoxicity

The genotoxicity of BD metabolites has been demonstrated in all experimental systems that provide for metabolism (reviewed in deMeester, 1988; Himmelstein et al., 1997; Pacchierotti et al., 1998). The mutagenic activity of the metabolites has been demonstrated many times over in the Ames Salmonella Test, where metabolic activation was achieved by the addition of S-9 fractions of liver from various species. DEB was the most potent mutagenic intermediate when the various intermediates were tested separately. BD metabolites have also induced gene mutations in lower eukaryotes and insects, as well as in cultured mammalian cells (Recio et al., 2000). In cultured human cells, the DEB metabolite was 100- and 350-fold more potent on a molar basis than the EB and EB-diol metabolites, respectively (Cochrane and Skopek, 1994).

The kinds of mutational changes induced by BD metabolites in vitro have been studied by analyses of mutational spectra. Early studies showed increased frequencies of A → T base substitutions, suggesting that adenine adducts have a greater mutagenic potential than guanine adducts. More recent studies, however, found base substitutions of guanine in addition to the changes at adenines (Recio et al., 2000). Of note, DEB, which also preferentially induces A → T base substitutions in human cells, also significantly increased the frequencies of large gene deletions (Recio et al., 2000). These spectra are consistent with the monofunctional and/or bifunctional alkylations expected by the EB and DEB metabolites, respectively.

Mammalian cells in culture, including human, have also shown increased levels of DNA damage following EB or DEB exposures (IARC, 1999). DEB was again the more potent of the two metabolites, with effective concentrations 10–50-fold lower than EB for inducing sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) and human lymphocytes, respectively. Detoxification of the BD metabolites is apparently important in preventing such damage. Lymphocytes from GST T1 null individuals (homozygous deficient for this isozyme) but not from GST M1 null individuals showed significantly higher frequencies of SCEs following DEB in vitro exposures. In contrast, lymphocytes from the GST M1 null individuals were more susceptible in vitro to SCE induction by EB (Uuskula et al., 1995) although neither GST T1 nor GST M1 genotypes affected SCE levels following in vitro exposures to EB-diol (Bernardini et al., 1996; Hayes et al., 2000a; Albertini et al., 2001, 2003).

In vivo mutation studies in experimental animals exposed to BD allow comparisons with animal carcinogenicity data and potential extrapolations to humans (Walker and Meng, 2000). Exposures of mice and rats to BD by inhalation have shown induction of somatic gene mutation (mostly at the *HPRT* locus) in vivo. In mice, the dose–response curves suggest that BD has greater mutagenic activity per ppm (this is also true for DNA adducts, see Swenberg et al., 2001) at low exposure levels than at high levels (Walker and Meng, 2000). In vivo studies using different BD metabolites have suggested that DEB is the in vivo metabolite responsible for BDs mutagenicity at low exposure concentrations. Furthermore, molecular analyses of *HPRT* mutations showed a statistically significant increase in large deletions in BD exposed mice, also consistent with DEB being the important mutagenic metabolite (Walker and Meng, 2000).

Studies of in vivo *HPRT* mutations in BD exposed rats in a multi-dose experiment (highest dose was 625 ppm; 1381.3 mg/m^3) also showed significant increases in *HPRT*, but with a mutagenic potency 8.5-fold

lower than in mice (Walker and Meng, 2000). DEB also induced *HPRT* mutations in rats at even higher frequencies than it did in mice, indicating that this metabolite is mutagenic in rats. However, direct administration of DEB eliminates the difference between the two species in the in vivo formation of this reactive metabolite.

Numerous investigators have demonstrated a significant increase in chromosome changes in vivo in blood cells of BD-exposed mice. Injection of BD metabolites directly into mice or rats produces chromosomal genetic changes in both species, but similar chromosome changes have never been demonstrated in vivo in rats following exposure to BD (reviewed in Pacchierotti et al., 1998).

The germ cells of rodents exposed to BD and/or its metabolites also show genotoxic effects. Dominant lethality, an expression of chromosome aberrations in germ cells, was shown in mice, but not in rats (Anderson et al., 1998). Again, based on similar effects when animals are treated with various metabolites of BD, this difference does not appear to be due to differential sensitivity between rat and mouse germ cells, but due to differences in the formation of these reactive metabolites.

In summary, BD metabolites EB, DEB, and EB-diol are mutagenic in multiple test systems, with potencies in the order of DEB $\gg$ EB $\gg$ EB-diol. HMVK would be expected to be mutagenic, but its potential relative to the other BD metabolites is not known. In vivo, both gene and chromosome level mutations are induced by BD in mice, but only DNA damage has been shown in rats following exposure to BD. Even for DNA adducts, BDs mutagenic potency is much greater in mice than in rats. In contrast to the parent BD, the metabolites of BD are mutagenic at both levels in vivo in both species. These observations are consistent with the results of metabolic studies, hemoglobin adduct data and the relative carcinogenicity of BD in the two species.

3.1.5. Mode of action as a carcinogen

K-ras and *H-ras* mutations have been found in the tumors of mice in the two-year cancer bioassays (Goodrow et al., 1990, 1994). The former are most significant as they were seen only in the BD-exposed animals and not in control animals. Allelotyping of lung and mammary tumors induced in the two-year bioassays has also revealed frequent genetic changes manifest as losses of heterozygosity, presumably due to deletions and/or translocations in regions of tumor suppressor genes (Wiseman et al., 1994). Together with the unequivocal evidence of genotoxicity for the reactive BD metabolite noted above, these observations support the hypothesis that genotoxicity is the mechanism responsible for BDs carcinogenicity and that large-scale genetic changes such as deletions are at least partially responsible.

3.1.6. Molecular epidemiological studies in humans

Biomarkers of BD metabolism in humans in an occupational setting have been measured by several investigators (see Table 1 and included references). The biomarkers have included measures of the urinary BD metabolites and HBVal and THBVal hemoglobin adducts. It has been calculated that HBVal adducts are produced at a rate of 0.004 pmol/g globin/hour by an average exposure to BD of 3.5 ppm (7.7 mg/m^3) (Osterman-Golkar et al., 1993). In a study of Chinese workers, THBVal adduct concentrations plotted against mean exposure levels for 33 workers and 25 controls (0 ppm) showed a regression slope significantly greater than zero ($r^2 = 0.33$) (Swenberg et al., 2000). Of note, the THBVal adduct concentrations in this and other studies were also present in non-BD exposed individuals, suggesting endogenous production of identical adducts.

DNA adducts in BD exposed workers have also been studied (Zhao et al., 2000). Workers in a monomer production facility with BD exposures ranging from $<$0.02 to 37.6 mg/m^3 had *N*l-(2,3,4-trihydroxybutyl)adenine (*N*1-THBAde) adduct levels ranging from $<$0.1 (limit of detection) to 25.0 adducts/10^9 nucleotides (mean of 4.5 adducts/10^9 nucleotides). By contrast, mean *N*1-THBAde levels were significantly lower in 11 unexposed controls. Adduct levels in exposed workers appeared to correlate with BD exposures.

Biomarkers of genotoxicity have also been investigated in BD exposed humans with inconsistent findings. Three studies from one laboratory of BD exposed workers in a Texas facility were positive, using the autoradiographic assay for *HPRT* mutations in blood lymphocytes, as was another study by this group, using the cloning assay. Mutational spectrum data were compatible with the *HPRT* mutations being induced by BD in that an excess of deletions were found. By contrast, two studies in other laboratories, using the cloning assay, failed to find increases in mutations in blood lymphocytes of BD exposed Chinese and Czech workers even though BD exposure concentrations were similar to those in the Texas studies. Furthermore, no increases in structural chromosome aberrations in mitogen-stimulated blood lymphocytes were found in the Texas workers, although an in vitro "Challenge Assay," possibly measuring unexpressed chromosome damage, was positive (Au et al., 1995). An in vivo study of European BD workers also failed to show evidence of BD-induced chromosome aberrations, SCEs or micronuclei on original analysis (Sorsa et al., 1994). However, re-analysis with respect to GST genotypes showed the GST Tl null BD exposed workers to have significantly higher structural aberration frequencies than the appropriate controls (Sorsa et al., 1994). A recent second in vivo study of these Czech workers did show significant elevations in chromosome aberrations and SCEs in blood lymphocytes of the BD exposed group (average 1.6–1.8 ppm,

Table 1
Results of molecular epidemiological studies of BD-exposed workers prior to the recent large Czech study

	Urine metabolites	Adducts		Somatic gene mutations		Chromosome mutations				
		Hemoglobin	DNA	Autorad[e]	Cloning	Chr. Abs.[a]	SCE[b]	Micro[c]	Aneuploidy	Challenge
Texas manomer production										
Study 1	M1; dose–response elevation (Ward et al., 1996)	Few workers show elevation (Osterman-Golkar et al., 1993)		+ Dose response (Ward et al., 1994)						BD group ↑ (Au et al., 1995)
Study 2	M1; ±Elevation (Ward et al., 1996)			BD group ↑ (Ward et al., 1996)						
Texas SBR workers				BD group ↑ (Ward et al., 1996; Ammenheuser et al., 2001)	BD group ↑ (mutation spectra consistent with BD) (Ma et al., 2000)					
Czech Republic										
Study 1		↑ HBVal in BD group (Sorsa et al., 1996)	↑ in BD group *N*1-THBAde (Zhao et al., 2000)		No changes (Tates et al., 1996)	No changes; + when reeval. by GST status (Sorsa et al., 1994, 1996)				
Study 2						↑ in BD group (Sram et al., 1998)	↑ in BD group (Sram et al., 1998)	No changes[d] (Sram et al., 1998)		
Chinese study		↑ THBVal in BD group (Swenberg et al., 2000)			No changes (Hayes et al., 2000)		No changes (Hayes et al., 2000)		No changes (Hayes et al., 2000)	

[a] Chromosome aberrations.
[b] Sister chromatid exchanges.
[c] Micronucleus assay.
[d] Also no changes by COMET assay.
[e] Autoradiography.

3.5–4.0 mg/m^3) compared to controls (Sram et al., 1998). However, the study of Chinese BD polymerization workers referenced above gave negative results for SCE and aneuploidy (Hayes et al., 2000a).

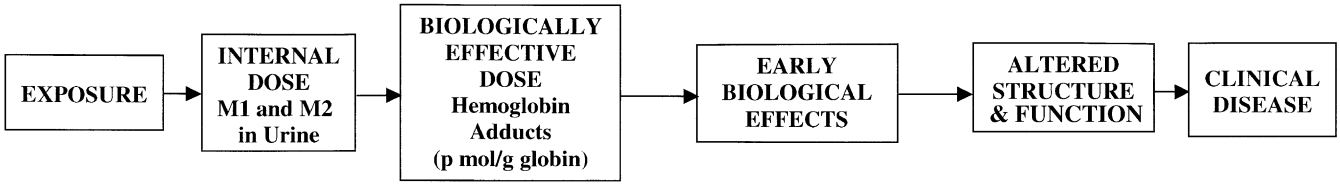
One difficulty with the above listed biomarker studies in humans has been the paucity of BD exposure information for the study populations. To remedy this situation, a recent large-scale transitional epidemiological study of Czech BD workers was undertaken of a worker population with extensive pre-study measurements of external BD exposures. An average of 10 independent eight-hour personal exposure measurements over a two-month period were made for each potentially exposed worker. In addition, ambient air BD levels and coexposures to toluene, styrene and benzene were measured. M1 and M2 urinary metabolites, as well as the HBVal and THBVal hemoglobin adducts were measured as biomarkers of BD exposure; several assays for gene impacts (*HPRT* mutation by two methods), DNA damage (SCE), and structural chromosomal aberrations [by conventional and fluorescence in situ hybridization (FISH) methods] were employed (Albertini et al., 2001) (Table 2). The study included 24 BD monomer production workers [mean BD exposure = 0.642 mg/m^3 (0.290 ppm)], 34 polymerization workers [mean BD exposure = 1.794 mg/m^3 (0.812 ppm)], and 25 controls [mean BD exposure = 0.023 mg/m^3 (0.010 ppm)]. Urinary M1 and M2 metabolite and HBVal and THBVal hemoglobin adduct concentrations were all elevated in exposed workers and significantly correlated with BD exposure levels, with adducts being the most highly associated. The ratio of urinary metabolites concentrations [M1/(M1 + M2)] was approximately 0.99, indicating that humans rely almost entirely on the hydrolytic detoxification pathway. THBVal adduct concentrations were 300–400-fold higher than HBVal adduct concentrations, presumably reflecting this hydrolytic pathway producing much greater in vivo concentrations of EB-diol than EB in humans. Background (control) THBVal adduct concentrations were higher than previously observed in other control populations (North American and Chinese), suggesting an endogenous source for the adduct. Despite documented external BD exposures and urinary metabolites and Hb adducts revealing appreciable internal doses in exposed workers, there was no evidence of genotoxicity in this study. Metabolic genotypes did not influence any of these findings except the degree to which conjugation contributed to BD detoxification in vivo.

In summary, all of the biomarkers studied in BD exposed animals have been assessed also in humans. Humans produce the EB and EB-diol metabolites in vivo and effectively hydrolyze them, as shown by M1/(M1 + M2) ratios in urine, to both HBVal and THBVal hemoglobin adducts, the latter being more prevalent. In this regard, humans are more like rats than mice. The evidence for BD-induced genotoxicity in vivo in humans remains inconclusive. However, at the BD exposure levels encountered in modern industry, the recent large study failed to demonstrate either induction *of HPRT* mutations or cytogenetic changes, even though measurable levels of electrophilic BD metabolites were produced in vivo. Studies of workers at higher exposure levels are required to clarify the question of genotoxicity, which is the important qualitative non-tumor endpoint for making human cancer risk assessments for BD.

3.1.7. Use of biomarkers for cancer risk assessment

Non-tumor data have now been obtained under comparable conditions for BD exposed mice, rats, and

Table 2
Partial results of the recent large study of biomarkers and responses in BD-exposed workers based on extensive exposure measurements



Exposure ppm	M1/(M1 + M2)	HBVal[a]	THBVal[a]	*HPRT* mutations[b]	Chromosome changes[c]	Cancer
0.010 ($N = 25$)	0.996	0.22 ± 0.21	95 ± 39	—	—	
0.290 ($N = 24$)	0.989	0.47 ± 0.45	179 ± 101	0[d]	0	?
0.812 ($N = 34$)	0.985	2.23 ± 1.40	716 ± 426	0	0	

M1—1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane; M2—1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene (mean concentration not shown).
Influenced by GST genotype.

[a] HBVal, *N*-(2-hydroxy-3-butenyl)valine; THBVal, *N*-(2,3,4-trihydroxybutyl)valine.

[b] *HPRT* mutations assessed by both cloning and autoradiographic assay methods; no change in *HPRT* mutational spectrum from background.

[c] Chromosome changes measured include sister chromatid exchanges (SCEs) and chromosome aberrations measured by conventional methods and by FISH.

[d] 0, No significant differences compared to background controls.

humans (Albertini et al., 2001, 2003; Walker and Meng, 2000; Meng et al., 2001; Swenberg et al., 2001; V.E. Walker, personal communication). While vastly different over their entire ranges, the levels of BD exposures in the three species have been comparable at the lower concentrations. These data might be used to begin modeling BD's mode of action in inducing cancer and for assessing human cancer risk (Table 3).

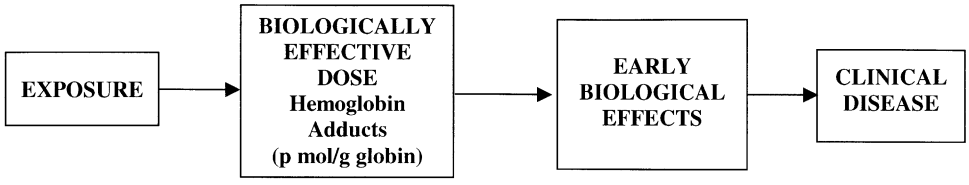
HBVal and THBVal concentrations provide measures of biologically effective internal doses in the three species. Mice show higher HBVal adduct concentrations (oxidative pathway) than rats at all external exposure levels, while THBVal adduct concentrations are comparable except at the highest external exposures. HBVal adducts in humans are considerably lower than in rodents, while THBVal adduct concentrations (hydrolysis) are much higher, reflecting the dominance of hydrolysis over oxidation in BD metabolism in humans. The dominance of hydrolytic detoxification in humans is also revealed by ratios for M1/(M1 + M2) concentrations in urine that show 24% hydrolysis in mice, 51% hydrolysis in rats, and 99% hydrolysis in humans.

The most important interspecies comparisons shown in Table 3 are for genotoxic effects which, in these rodent studies, are best represented by *HPRT* mutations (cloning assay). Mice showed statistically significant increases in mutations over controls even at exposure levels as low as 3 ppm administered by inhalation for two weeks, confirming that mice are exceedingly susceptible to the genotoxicity of BD. Induced *HPRT* mutations were not seen in rats until external BD exposure levels of 625 ppm were achieved, and even then the exposures had to be continued for four weeks to show the effect. In contrast, humans showed no increases in *HPRT* mutations over background in the only study where there was good external exposure assessment.

These comparative results can be considered in the context of other studies (reviewed in Albertini et al., 2001). As noted above, chromosome aberrations have been frequent in vivo observations in mice exposed to BD, but have never been observed in rats. The transitional study in Czech BD workers also included several assays for DNA and chromosomal damage; all were unequivocally negative (Albertini et al., 2001, 2003).

Data gaps become apparent when viewing BD nontumor data in this way. The animal studies were all conducted in traditional fashion (i.e., exposures at various levels followed by removal of the agent), allowing time for development of biomarkers, and then testing. The animals should have been continuously exposed through the time of testing to allow their biological responses to be determined under conditions that are exactly comparable to exposed humans. In addition, before we can accurately determine the significance of the various metabolic pathways in rats, mice, and humans (including gender differences), we will need comparative data on adducts that are specific for each of the four electrophilic metabolites of BD, and such data are not yet available. There is a lack of human studies at BD exposure levels >2 ppm and, at present, a critical noncancer early biological effect endpoint, i.e., genotoxicity, has not been found with certainty in humans, at the

Table 3
Minimal effective or maixmal no-effect BD exposure levels in mice, rats, and humans



ppm	HBVal[a]	THBVal[a]	HPRT mutations	Cancer
Mice				
3	38 ± 22	353 ± 104	↑[c]	+[e]
Rats				
62.5	85.0 ± 4.0	2990 ± 150	0[d]	NT[f]
625	ND[b]	ND	↑	NT
Humans				
0.796	2.23 ± 1.40	716 ± 426	0	?

[a] HBVal, *N*-(2-hydroxy-3-butenyl)valine; THBVal, *N*-(2,3,4-trihydroxybutyl)valine.
[b] ND, not determined.
[c] ↑, Statistically significant increase over background controls (Meng et al., 2001).
[d] 0, No statistically significant increase over background controls.
[e] Positive for lung tumor induction in females at the lowest exposure level tested (6.25 ppm) in long-term bioassay in mice (Huff et al., 1985; Melnick et al., 1990).
[f] NT, not tested in long-term bioassay in rats (Owen et al., 1987).

exposure levels examined. Clearly, if there are human populations exposed to higher levels, they should be studied with all relevant biomarkers in an attempt to document the extent of associated genotoxicity. Detection of increased levels of genotoxicity would allow the above paradigm to be used to quantitatively extrapolate the risk for cancer in humans, using the combined human and animal datasets with the animal cancer results. Also, finding a level of exposure to BD that results in evident genotoxicity in humans would specify critical concentrations for biomarkers of exposure, e.g., hemoglobin adducts that indicate biologically effective doses. The HBVal adduct concentration might be the critical marker of effective exposure. However, if genotoxicity is not found in humans despite the presence of increased levels of hemoglobin adducts, whether or not BD is effectively genotoxic (and thus carcinogenic) in humans at exposure levels likely to be experienced must be questioned.

3.2. Vinyl chloride

Vinyl chloride monomer (VC) is a highly volatile organic compound used as an intermediate in the production of polyvinyl chloride. Worldwide production was approximately 27 million tons in 1998. The U.S. EPA has classified VC as a known human carcinogen based on a causal association between inhalation occupational exposure to VC and development of angiosarcoma; consistent evidence of carcinogenicity in rats, mice, and hamsters by both the oral and inhalation routes; and mutagenicity and DNA adduct formation by VC and its metabolites. IARC has also concluded that sufficient evidence exists for classification of VC as Group 1 (carcinogenic to humans). VC is used to illustrate the use of non-tumor data to (1) define the mode of action, (2) develop a measure of internal dose, and (3) improve understanding of the dose–response curve.

3.2.1. Cancer studies

Concern about VC carcinogenicity began in 1974, when it was reported that three workers at a single VC polymerization plant were diagnosed with hepatic angiosarcoma (Creech and Johnson, 1974), an exceedingly rare cancer in humans. Subsequently, multiple epidemiologic studies demonstrated an association between exposure to VC and angiosarcoma of the liver. Also numerous experimental studies in rats and mice demonstrated that VC is a liver carcinogen by both the oral and inhalation routes. There is no indication that VC causes liver tumors via a cytotoxic mechanism; thus, necrosis is not considered a preneoplastic effect. After recognition as a human carcinogen, occupational exposure to VC was drastically reduced, but VC still remains of concern in occupationally exposed workers and also because of its presence in the environment as a microbial degradation product of trichloroethylene and perchloroethylene (Kielhorn et al., 2000).

3.2.2. Mode of action

The liver is clearly the primary target organ for VC-induced cancer as evidenced by the rare tumor type (liver angiosarcoma) occurring in both human and animal populations. VC carcinogenicity occurs via a genotoxic pathway that is at least partially understood. VC is metabolized to a reactive metabolite, chloroethylene oxide (CEO) (Fig. 2). The reactive metabolite binds to DNA, forming DNA-adducts that lead to mutations, uncontrolled cell growth, and tumor formation.

3.2.3. Metabolism

Numerous studies have been conducted on the pharmacokinetics and metabolism of VC, primarily in rats. The primary route of metabolism of VC is via a CYP450 2E1-mediated pathway (Watanabe et al., 1976; Buchter et al., 1980) to form CEO. This highly reactive epoxide then spontaneously rearranges to 2-chloroacetaldehyde. While 2-choroacetaldehyde does form protein adducts, it is not thought to be mutagenic. Saturation of the CYP2E1 metabolic pathway occurs in rats, monkeys, and humans at levels of exposure consistent with the plateau of hepatic angiosarcoma incidence in rat bioassays.

3.2.4. Molecular dosimetry

CEO reacts with DNA in vitro to form 7-(2-oxoethyl)guanine and a variety of exocyclic base adducts (Guengerich, 1992; Guengerich and Persmark, 1994) (Fig. 2). Although 7-(2-oxoethyl)guanine is by far the major adduct formed in vitro, this adduct does not appear to be mutagenic (Barbin et al., 1985). Therefore, most research has focused on the minor exocyclic adducts. The formation, role, and detection of these adducts has been reviewed (Bartsch et al., 1994).

The formation of 1,N^6-ethenoadenine, 3,N^4-ethenocytosine, and N^2,3-ethenoguanine (N^2,3-eGua) adducts following acute exposure to VC has been extensively characterized in rats (Swenberg et al., 1999; Barbin, 1999). These adducts are biomarkers of exposure at the genetic level and are widely believed to be relevant to VC-induced carcinogenesis. However, these single-exposure studies are of limited use to quantitative risk assessment.

3.2.5. Mutagenicity

Several types of experimental studies indicate that VC metabolites are genotoxic. VC itself is not directly mutagenic, but is positive in a variety of assays that allowed for metabolic activation. The reactive VC metabolite, CEO, is genotoxic without activation. The molecular epidemiology of VC supports the conclusion that it is a genotoxic carcinogen. Elevated levels of micronuclei, SCE, and chromosome aberrations in blood lymphocytes; single-strand breaks in liver DNA, and mutations

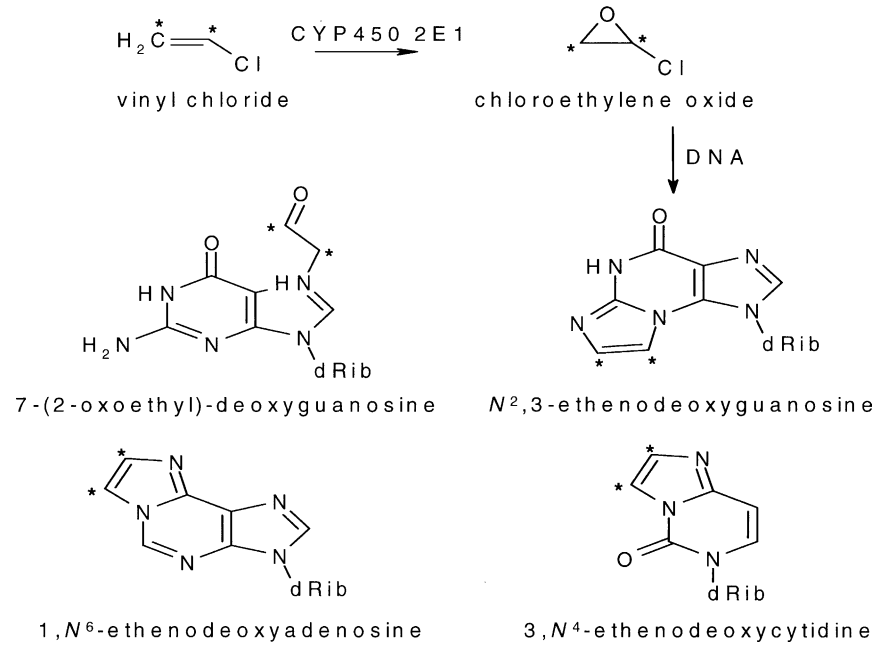


Fig. 2. Adducts induced in rats exposed to VC. In the case of [$^{13}C_2$]VC exposures, (*) denotes position of labeled atoms.

in oncogenes have been noted in occupationally exposed workers. Smith et al. (1998a,b) reported a dose-dependence relationship between VC exposure and increased mutant p53 proteins in 225 French VC workers. When p53 was examined in tumors of VC workers, A → T transversions were found in two of four tumors at the first base of codon 249 or 255 (Hollstein et al., 1994). The same investigators found a transforming G → A transition at codon 13 of the c-Ki-*ras*-2 oncogene in 15 of 18 ASL (Marion et al., 1991). An exposure-dependent increase in DNA single-strand breaks and alkali-labile sites in lymphocytes was recently reported for Egyptian VC workers (Awara et al., 1998).

3.2.6. Pharmacokinetic modeling

VC illustrates a classic problem in risk assessment: although VC is a strong human carcinogen and multiple human epidemiologic studies exist, exposure data associated with most of these studies are inadequate to derive risk estimates. Thus, EPA based its human risk estimates on animal data. Risk estimates were calculated with the aid of a PBPK model (Clewell et al., 1995), using a dose metric of daily active metabolite generated per unit of liver volume. In the model, VC metabolism was hypothesized to occur via two pathways, one representing oxidation by cytochrome P4502E1 and the other oxidation by other isozymes of P450. As detailed in the EPA document (U.S. EPA, 2000), the model successfully simulated pharmacokinetic data from a large number of studies over a wide range of concentrations and multiple routes of exposure.

The inhalation risk was based on the incidence of liver angiosarcomas, angiomas, hepatomas, and neoplastic nodules in female Sprague–Dawley (SD) rats in the carcinogenicity bioassay of Maltoni et al. (1981). The use of the PBPK model allowed the inclusion of exposure concentrations from 1 to 6000 ppm since the PBPK dose metrics linearized the dose–response relationship. The PBPK model was used to calculate a human equivalent dose of metabolites to the human liver based on the rat exposures. Because VC metabolism is linear in the human dose range of interest, this equivalence factor was used to convert the risk based on the dose metric (now in humans) into the human oral dose. In accordance with the established guidelines, the linearized multistage model was used to calculate an inhalation unit risk estimate of 4.4×10^{-6} per $\mu g/m^3$. A 2-fold increase to 8.8×10^{-6} per $\mu g/m^3$ factor was recommended to account for the additional sensitivity of young populations to a continuous lifetime exposure. The resulting inhalation unit cancer risk was 84-fold lower than those calculated under the previous risk assessment guidelines (U.S. EPA, 1999). The incorporation of pharmacokinetic parameters accounted for a factor of 48; the use of the expanded dataset (Chen and Blancato, 1989) accounted for the remaining factor of approximately 2.

3.2.7. DNA adducts

A recently completed study (Morinello et al., 2002a) based on formation of the promutagenic adduct N^2,3-eGua provides the exposure–response characterization necessary for consideration in risk assessment. Twelve week-old male SD rats were exposed in a whole body inhalation apparatus to 0, 10, 100, or 1100 ppm VC for 1 or 4 weeks (6 h/day; 5 days/week). To allow the study of adducts in different liver cell populations, the livers were

perfused in situ with collagenase (Johnson et al., 1999), and hepatocytes were separated from nonparenchymal cells by centrifugation (Lindamood et al., 1982).

The concentration of N^2,3-eGua in the hepatocytes from each animal was measured with a highly specific immunoaffinity/gas chromatography/high resolution mass spectrometry assay (Ham et al., 1999). In each case, the exposure–response curve displays a supralinear pattern, which reflects the saturation of metabolic activation of VC between 100 and 1100 ppm. There was a 2–3-fold accumulation of the N^2,3-eGua between 1 and 4 weeks at the two highest exposure concentrations.

In order to study the repair of N^2,3-eGua, a group of rats was similarly exposed to VC for four weeks, but was allowed to recover for one week following exposure before they were killed. The absence of a decrease in adduct concentrations following the recovery period indicates that N^2,3-eGua is repaired slowly in vivo. This finding is consistent with the nearly linear increase in adduct concentrations measured in SD rats exposed to 600 ppm VC for 1, 2, 4, or 8 weeks (4 h/day; 5 days/week) (Morinello et al., 2002a). The apparent persistence of this mutagenic lesion increases the probability that it is causally involved in VC-induced carcinogenesis in the rat.

3.2.8. Target organ susceptibility

As discussed in detail by Doll (1988), there is conflicting evidence in the literature regarding the possible association between occupational exposure to VC and brain tumors. Based on the well-established ability of VC to induce liver tumors in humans, brain tumors were excluded from the recent EPA VC risk assessment (U.S. EPA, 2000). The validity of this choice was evaluated by measuring N^2,3-eGua in rats exposed to VC or [$^{13}C_2$]VC (Morinello et al., 2002b). In the latter case, the mass spectrometry method allowed endogenous N^2,3-eGua to be differentiated from [$^{13}C_2$]N^2,3-eGua derived from the labeled VC. [$^{13}C_2$]N^2,3-eGua was clearly induced in DNA from the liver, but was absent in DNA of brain cells of the same rat. (The results from exposure to unlabeled VC were similar.) In addition, Barbin et al. (1985) reported that neither 1,N^6-ethenoadenine nor 3,N^4-ethenocytosine were induced in the DNA of brain cells of SD rats exposed to 500 ppm VC by inhalation for 8 weeks (4 h/day; 5 days/week). Taken together, these data provide strong evidence that VC-induced adducts do not contribute to carcinogenesis in the brain and provide a scientific justification to the agency's decision to exclude brain tumors from the risk estimate calculations.

3.2.9. Age-dependent susceptibility

It is often assumed that young animals and humans are more susceptible to chemically induced carcinogenesis than adults under the same conditions. To examine age-dependent differences in adduct formation, 25-day-old male SD rats were exposed to 0, 10, 100, or 1100 ppm VC for five days (Morinello et al., 2002a). The concentration of N^2,3-eGua in weanlings was 2–3-fold greater than measured in adults exposed for the same duration and was similar to that measured in adult rats exposed for four weeks. The angiosarcoma incidence after short-term, early life exposure is approximately equal to that of long-term exposure starting after maturity. Based on these observations, EPA assumes that continuous lifetime exposure from birth would about double cancer risk; however, currently, there are only limited data in humans regarding the comparative expression of CYP2E1 in children and adults.

3.2.10. Endogenous DNA adducts

Low concentrations of N^2,3-eGua and other exocyclic adducts have consistently been detected in unexposed animals and humans by several laboratories (Chaudhary et al., 1994; Mitro et al., 1995; Nair et al., 1995; Nath et al., 1996). It is believed that oxidative metabolic processes are at least partially responsible for this background presence. The formation of N^2,3-eGua, 1,N^2-eGua, 1,N^6-eAde, 3,N^4-eCyt, and related adducts from the reaction of the lipid peroxidation product trans-4-hydroxy-2-nonenal or its epoxide have been demonstrated (el Ghissassi et al., 1995; Sodum and Chung, 1998, 1989; Chen et al., 1998; Ham et al., 2000; Morinello et al., 2000; Morinello and Swenberg, 2000). Since these adducts are identical to those derived from VC, they presumably have the same mutagenic potential. It has been proposed that the presence of such endogenous adducts may play a role in spontaneous carcinogenesis.

The mean endogenous N^2,3-eGua concentration measured in the 22 control adult rats in the exposure–response study was 0.46 ± 0.28 N^2,3-eGua/10^7 Gua. A significantly higher concentration ($p < 0.001$) of N^2,3-eGua, 1.7 ± 0.2 adducts/10^7 Gua, was measured in six samples of transplant-quality human liver from donors with no known exposure to VC. Notably, the concentration of N^2,3-eGua measured in the human liver samples is higher and less variable than that measured in any rodent model to date using this methodology.

3.2.11. Implications to risk assessment

The molecular dosimetry data, which are indicative of the effects of VC at the genetic level, suggest that metabolism is predictive of VC-induced mutagenesis. These data therefore provide a sound mechanistic justification for the use of PBPK modeling in the risk assessment of VC and other well-characterized genotoxic carcinogens.

Despite the large advances recently made in our understanding of VC molecular dosimetry, there remain significant obstacles that currently limit application of these data to quantitative risk assessment. The most significant shortcoming is the lack of adduct formation

and repair data in humans exposed to VC. Without this information, it is problematic to make the appropriate animal-to-human extrapolations required for use in quantitative risk assessment. Future studies of adduct formation in surrogate tissues, such as lymphocytes from VC-exposed workers, may help to complete these knowledge gaps. Also, since VC induces the formation of at least four adducts in vivo, it will be necessary to better characterize the relative contribution of each to VC-induced carcinogenesis.

The presence of endogenous etheno adducts in humans has important implications to contemporary risk assessment. For example, exposure of rats to 10 ppm VC for four weeks resulted in a 5-fold increase over the endogenous concentration of N^2,3-eGua. A linear interpolation suggests that exposure to 1 ppm would result in only a 5% increase over the endogenous N^2,3-eGua concentration. Since humans have an even higher level of background adduct levels, this finding may explain why no cases of hepatic angiosarcoma have been reported in VC workers who began employment after adoption of the 1 ppm TLV.

The increased susceptibility of weanling rats to VC-induced adduct formation is consistent with the relatively high incidence of hepatic angiosarcomas and hepatocellular carcinomas in young rats (Maltoni et al., 1981; Maltoni and Cotti, 1988). The concentration of N^2,3-eGua adducts measured in weanlings is generally two to three times greater than in adults for the same exposure duration. It is possible that greater adduct concentrations, coupled with higher cell proliferation rates in the young animals, underlie the increased tumor incidence. These data help to justify the inclusion of a protection factor for young populations in the recent EPA risk assessment of VC. Taken alone, the N^2,3-eGua data suggest that the 2-fold protection factor is biologically appropriate.

3.2.12. Discussion

Since it is one of the most comprehensively studied human carcinogens, VC is a good model with which to evaluate the effects of the inclusion of mechanistic data in the risk assessment of genotoxic carcinogens. Given our understanding of VC metabolism in the rat and human, the use of a PBPK model to make extrapolation between species and routes of exposure was appropriate in this case and marked a significant step towards the inclusion of additional scientific data in risk assessments.

Despite the value of DNA adducts as relevant and useful biomarkers of effect, the consideration of these data in quantitative terms remains problematic. Since VC is known to induce several adducts, the relevance of each to carcinogenesis must first be established. It will also be necessary to obtain human data that will allow appropriate interspecies extrapolations to be made. It is hoped that additional research can fill these knowledge gaps and will make the inclusion of such molecular dosimetry in the quantitative risk assessment of genotoxic carcinogens feasible in the near future.

The adduct data presented are valuable because they help to justify several of the choices made by the EPA in the recent VC risk assessment. The adduct data demonstrate the relevance of metabolic parameters to VC-induced mutagenesis and support the use of the PBPK model. They also support the inclusion of a protection factor for young populations in the risk assessment, as well as the exclusion of brain tumors from the calculations. The VC case study illustrates how even incomplete non-tumor data can effectively increase confidence in a tumor-based risk assessment.

3.3. Benzene

Benzene (BZ) is a highly volatile, flammable hydrocarbon used in the manufacture of plastics, detergents, pesticides, and other chemicals and is a natural component of crude oil and gasoline. BZ is widely used in the United States; it ranks in the top 20 chemicals in production volume. The U.S. EPA has classified BZ as a known human carcinogen based upon both human and animal evidence. Epidemiologic studies provide evidence for a causal association between exposure to BZ and leukemia. Additionally, changes in blood and bone marrow consistent with hematotoxicity are recognized in humans and experimental animals. We use BZ to illustrate (1) the use of non-tumor data to define the mode of action and (2) the use of internal measures to define dose to target tissue.

3.3.1. Leukemogenesis

Leukemias are monoclonal malignancies of the circulating blood cells, the majority of which originate from individual stem or progenitor cells in the bone marrow. Leukemias are classified as ''myeloid'' or ''lymphoid'' depending on whether the phenotype of the abnormal clone possesses characteristics of either of these major lineages of blood cells. If the malignant cell clone possesses some differentiating or maturational ability, the leukemia is classified as ''chronic.'' Alternatively, if undifferentiated cells predominate, the disease is classified as ''acute.'' Based largely on morphology and phenotype, leukemias are classified as chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and acute myeloid leukemia (AML), as well as individual subtypes within each category. The myelodysplastic syndromes (MDSs) are also life-threatening monoclonal bone marrow abnormalities that may transform to AML. Benzene exposure is an established cause of AML and MDS, and has also been associated with other forms of leukemia (Hayes et al., 1997, 2000b; Savitz and Andrews, 1997).

AML involves the neoplastic proliferation of cells of myeloid lineage (i.e., granulocytic, monocytic, megakaryocytic, erythroid), with subtypes (M0–M7) based on morphologic criteria that distinguish undifferentiated abnormal myeloid cells from those that exhibit features typical of the individual lineages (Bennett et al., 1985). Consistent with present models for the origin and progression of neoplasia, AML is recognized to represent the end stage in a multistep progression in which multiple independent genetic alterations in proto-oncogenes or suppressor genes, together with epigenetic and environmental factors, contribute to the development of the full malignant phenotype (Alcalay et al., 2001; Mecucci et al., 2002).

A hallmark of leukemias in general and MDS/AML in particular is the involvement of specific clonal structural or numerical chromosome aberrations (CA) (Sandberg, 1990). Aneuploidy (numerical aberrations) features prominently in the development of these neoplasms. Monosomy 7, monosomy 5, and trisomy 8 are distinct clonal lesions associated with the development of MDS/AML (Sandberg, 1990). Trisomy 21, the congenital chromosome abnormality occurring in Down's syndrome, carries with it an increased risk of AML (Taub, 2001). Interstitial deletions, primarily involving chromosomes 5 or 7 (e.g., del(5q31) and del(7q)), are also observed in both MDS and AML. Other non-random chromosomal abnormalities encountered in AML include reciprocal translocations such as t(8;21) or t(15;17) and balanced translocations involving common breakpoints such as 11q23 and 3q26 (Sandberg, 1990). It should also be noted, however, that point mutations in the RAS proto-oncogene are also common in AML and MDS being found in 25–30% of cases (Beaupre and Kurzrock, 1999).

The results of these and numerous other studies indicate that clonal CA, including aneuploidy and interstitial deletions involving specific chromosomes, are important factors in the development of AML. In t-MDS/t-AML numerical or structural CA involving specific chromosomes are the earliest structural events that have been associated with the development of the disease (Pedersen-Bjergaard, 1995). Moreover, genes known to be important in the regulation of hematopoiesis have been repeatedly identified within or close to critical deleted chromosomal regions in MDS/AML, particularly 5q31 (Pedersen, 1996; Zhoa et al., 1997).

3.3.2. Mechanisms involved in BZ leukemogenesis

It is generally agreed that many of the molecular changes found in BZ-induced leukemia clones result from structural or numerical chromosomal damage to bone marrow stem cells during leukemogenesis. Evidence that BZ and/or its metabolites can cause such changes is reviewed below. How non-mutational events contribute to leukemogenesis is less clear. Cytotoxicity, induction/suppression of apoptosis, epigenetic alterations in gene expression, and modulation of intercellular signaling are likely to be involved in destabilization of the bone marrow. However, interpretation of these events requires an understanding of bone marrow biology that is still evolving. Genetic damage is perhaps a more easily measured endpoint.

3.3.3. Metabolism

BZ is metabolized in the liver by cytochrome P4502E1 (CYP2E1) to its major metabolites: phenol, hydroquinone, and catechol. The intermediate benzene oxide can also undergo ring opening to *trans*,*trans*-muconaldehyde. Although benzene metabolism is required for toxicity and carcinogenic response, the specific combination of metabolites involved is unknown, although it is thought that benzene-induced myelotoxicity and genotoxicity result from a synergistic combination of phenol with hydroquinone, muconaldehyde, or catechol.

3.3.4. Genotoxic mechanisms

BZ is able to induce many different kinds of genetic damage (e.g., point mutations, DNA adducts, oxidative DNA damage, structural CA, numerical CA, micronuclei, transformation, etc.) in a variety of in vitro and in vivo systems.

3.3.5. Direct DNA damage

A number of in vitro studies have been conducted which demonstrate the ability of benzene and/or various metabolites to induce DNA damage (e.g., strand breaks, alkali-labile sites, DNA adducts). DNA adducts have also been reported in cells of the bone marrow, liver and blood in mice exposed to benzene (Bodell et al., 1996; Li et al., 1996; Pathak et al., 1995). Based on in vitro studies, hydroquinone was concluded to be the primary metabolite responsible for adducts (Bodell et al., 1996; Pathak et al., 1995). Furthermore, a direct correlation between the extent of adduct formation and bone marrow toxicity was reported. Turtletaub and colleagues report that adduction to DNA occurs at very low doses of BZ and the dose–response curve for adduct formation appeared linear over the dose range tested (Creek et al., 1997). The significance of DNA adduct formation to persisting DNA damage is not yet known.

3.3.6. Mutations

BZ and its metabolites are relatively non-mutagenic in mutational assays specific for point mutations (e.g., *Salmonella typhimurium* reverse mutation assay; CHO-HPRT assay), while more active in mutational assays which detect large deletions as well as point mutations (e.g., mouse lymphoma $TK^{-/+}$ assay). BZ is also active in three mutation detection systems designed by Scheistl and colleagues (Aubrecht et al., 1995; Schiestl et al., 1997). The mutagenic activity of BZ has been evaluated

in the Big Blue mouse mutational assay. In homozygous lambda lacI transgenic Big Blue mice of the B6C3F1 strain, oral exposure to BZ in dose groups of 4–5 mice resulted in a dose-dependent increase in mutations in the spleen and bone marrow (Provost et al., 1996).

In humans, exposure to BZ resulted in a significant increase in mutations at the glycophorin A locus (Rothman et al., 1995). Most of the observed mutations were conversions from the heterozygous state to homozygosity. These presumably resulted from the loss of one allele followed by duplication of the other allele through recombination. In contrast, gene-inactivating events resulting in hemizygous phenotypes were not correlated with exposure. Glycophorin mutations occur in precursor cells of the erythrocytic lineage, and thus assess damage occurring in the bone marrow. These data are consistent with a mechanism involving chromosomal rearrangement in bone marrow cells.

3.3.7. Structural chromosomal aberrations

Several lines of experimental evidence indicate that BZ and/or its metabolites are clastogenic (i.e., capable of inducing structural chromosomal aberrations). The most compelling evidence is the number of studies reporting an increased frequency of chromatid- and chromosome-type aberrations in mitogen-stimulated peripheral blood lymphocytes sampled from currently exposed, healthy workers (reviewed in Zhang et al., 2002). Many in vitro and in vivo animal studies support the conclusion that exposure to BZ is associated with measurable chromosomal damage. In a recent occupational study, BZ exposure was associated with increased frequency of a specific chromosomal translocation, t(8;21) in the peripheral blood of exposed workers (Smith et al., 1998a).

3.3.8. Numerical chromosomal aberrations

Results of studies using the micronucleus assay suggest that BZ exposure may produce numerical CA. Several types of experiments in cultured cells confirm this activity. Studies of peripheral blood cells from healthy humans occupationally exposed to BZ have also produced evidence of aneuploidy (Zhang et al., 2002; Smith et al., 1998a). The molecular changes observed in secondary leukemia are consistent with the genetic activity profile for BZ and/or its metabolites: BZ is able to produce the aneusomies and chromosomal rearrangements that are commonly found in leukemic cells (Zhang et al., 2002). What remains to be determined is which metabolite or combination of metabolites poses the greatest risk of leukemia due to benzene exposure.

3.3.9. Cytotoxicity

Analyses of hematological parameters from cohorts of exposed workers have identified BZ-associated changes in peripheral blood cell populations. In the Pliofilm occupational cohort, reduced white blood cell count was associated with exposure to BZ at concentrations as low as 10 ppm (Ward et al., 1996b). Depression of lymphocyte count has also been observed in other cohorts (Moszczynski and Lisiewicz, 1982; Rothman et al., 1996). Peripheral neutropenia and loss of bone marrow progenitor cells was observed in a large Brazilian steel-worker cohort (Ruiz et al., 1994). In a group of highly exposed shoe workers, erythrocytes were found to be the most sensitive peripheral cell population (Bogadi-Sare et al., 1997). Thus, a number of occupational studies show that highly exposed workers are subject to reduced cell counts in the peripheral blood.

In patients who exhibit symptoms of BZ toxicity (peripheral neutropenia), bone marrow analysis generally supports the peripheral findings. That is, hypoplastic and dysplastic features of hematopoietic marrow are commonly noted (Ruiz et al., 1994; Bogadi-Sare et al., 1997; Aksoy et al., 1972; Forni et al., 1971). For example, in one series, abnormalities of the bone marrow such as hypoplasia and decreased myeloid:erythroid ratio were seen in approximately 85% of cases (Ruiz et al., 1994). Analysis of bone marrow from exposed experimental animals indicates cytotoxicity to hematopoietic cells at high doses (reviewed in ATSDR, 1997). Further, the cells of the bone marrow microenvironment, which are required for survival and differentiation of hematopoietic cells, exhibit BZ-induced toxicity.

In conclusion, BZ has been associated with cytotoxicity in a variety of blood and bone marrow cell types. The data on cytotoxicity are important because BZ poisoning, as defined by reduced WBC and/or platelet count, was found to be a significant risk factor for BZ-induced leukemia in a retrospective cohort study (Rothman et al., 1997). Whether all BZ-induced leukemias occur via a hematotoxic pathway is a matter of debate (Smith and Fanning, 1997), but the retrospective study discussed above found that only a fraction were associated with hematotoxicity.

3.3.10. Epigenetic mechanisms

In addition to overt cellular toxicity, BZ may have more subtle effects on hematopoietic development that destabilize the bone marrow tissue and promote disease conditions without directly damaging DNA. Exposure to hydroquinone in vitro alters the proportion of hematopoietic progenitor cells responding to stimulation with granulocyte-macrophage colony stimulating factor (Irons and Stillman, 1996). A possible mechanism underlying these results is stimulation of aberrant gene expression by reactive oxygen species (ROS). BZ's phenolic metabolites are further metabolized in the bone marrow to reactive free radical species. Redox cycling of these free radical species produces active oxygen, which, in addition to causing DNA damage, can induce altered patterns of gene expression in genes without structural damage. In turn, alterations in the normal pattern of

gene expression could alter differentiation pathways of hematopoietic cells and have profound effects on intercellular communications in the marrow environment. Such developmental effects may occur in the absence of overt toxicity or cell killing. With chronic or high dose exposure to BZ, the ability of bone marrow to compensate for benzene-induced disruption in development may be overwhelmed.

Epigenetic effects of this sort have been investigated by Irons and colleagues. They have shown that hydroquinone can induce changes in the developmental fate of bone marrow stem cells in culture (Irons and Stillman, 1996; Irons et al., 1992). Alterations in cell fate could lead to destabilization of bone marrow hemostatic mechanisms, thus promoting dysplastic conditions.

3.3.11. Non-tumor data available for BZ risk assessment

Based upon our understanding of the mechanistic processes involved, several biomarkers are of potential value in risk assessment for BZ-induced leukemia. These biomarkers can be classified into three types, potentially playing different roles in risk assessment. Biomarkers of exposure and/or results of pharmacokinetic modeling could replace ambient exposure levels as measures of dose. Biomarkers of early effect could be used as surrogates for leukemia incidence in dose–response modeling. Information on interindividual variability in susceptibility could be used to guide risk management interpretation of population risk estimates. Advantages and limitations of applying BZ biomarkers in risk assessment are discussed below.

3.3.11.1. Biomarkers of exposure. Several BZ exposure markers have been developed for the purposes of occupational monitoring.

BZ in blood and exhaled air of exposed persons reflects not only the external concentration of benzene, but also absorption and elimination dynamics. These measures thus provide information on the internal dose of BZ. Several studies provide data on the relationship between ambient air concentration and blood BZ levels (Berlin et al., 1980; Pekari et al., 1992; Money and Gray, 1989). However, exhaled air is not thought to be a reliable biomarker at low exposure levels (Money and Gray, 1989) and thus is not useful for dose scaling in cancer risk assessment.

Measurement of metabolic products in body tissues and fluids can help determine the relationship between ambient exposure levels and the resulting internal doses of actual carcinogenic species. The two markers thought to be the most sensitive to BZ exposure are urinary muconic acid and *S*-phenylmercapturic acid (S-PMA), able to detect exposure levels of 1 and 0.3 ppm, respectively (Ducos et al., 1992; Boogaard and van Sittert, 1995), although S-PMA appears to be superior (Boogaard and van Sittert, 1995). These minor metabolites are more specific and sensitive at low exposure levels than the phenolic products, due to high background levels of the latter. Both muconic acid and S-PMA exhibited a linear relationship with benzene exposure in several studies (Ducos et al., 1992; Boogaard and van Sittert, 1995; Bechtold et al., 1991; Ghittori et al., 1995; Gobba et al., 1997; Bechtold and Henderson, 1993), over the exposure ranges analyzed. Interindividual variability of urinary muconic acid concentration has also been well-studied and could be an indicator of differential susceptibility (Ducos et al., 1992; Gobba et al., 1997). However, muconic acid is also formed by sorbic acid, a common food additive, compromising the specificity of this marker.

Measures of macromolecular adduct formation have also been explored for use as benzene exposure biomarkers. DNA adducts have not yet been shown to be a useful biomarker in humans and their sensitivity in animal models has not been consistent. However, protein adducts appear to hold more promise. Early efforts to detect *S*-phenylcysteine (SPC) adducts formed by the reaction of benzene oxide to albumin and hemoglobin were a mixed success. The albumin adduct could be detected in exposed workers, but not the globin adduct (Bechtold et al., 1992; Rappaport et al., 1996). SPC adducts may be a good indicator of overall dose of toxic metabolites, since benzene oxide is formed by the primary oxidation of BZ and is thus a parent intermediate for production of the range of potentially leukemogenic secondary metabolites.

In recent years the methodology for detecting adducts of benzoquinone and benzene oxide to both albumin and hemoglobin has been improved (Rappaport et al., 2002; Bechtold and Strunk, 1996), providing the hope of greater sensitivity. The new methods have recently been applied to human samples from the Chinese studies (Rappaport et al., 2002). The data shows that the albumin and hemoglobin adducts of benzene oxide are highly correlated with BZ exposure. These measures hold considerable promise and deserve further study at lower levels of exposure.

The lack of certainty about which metabolites or combination of metabolites are the most carcinogenic and the lack of human data correlating exposure to BZ with metabolite concentrations in the bone marrow prevents incorporating exposure–dose corrections into dose–response modeling at this time. Current understanding of metabolism is still useful for risk assessment, since these biomarkers provide information on interindividual variability in the population.

3.3.11.2. Biomarkers of susceptibility. In occupational cohorts, only a small percentage of individuals with a similar exposure to BZ actually develop leukemia. Some genetic factors governing inter-individual differences in susceptibility have been identified and can be assessed in human populations.

BZ is oxidized in the liver primarily by CYP2E1, forming benzene oxide. Mice homozygous for a deletion of the CYP2E1 gene are not susceptible to the hematotoxic and genotoxic effects of BZ, indicating the importance of this enzyme in BZ toxicity (Valentine et al., 1996). A case-control study of workers with BZ poisoning assayed for CYP2E1 activity found an increased risk of BZ poisoning for fast hydroxylators as compared to slow hydroxylators, with an odds ratio of 2.5 (1.1–6.0 95%CL; adjusted for age, sex, BMI, alcohol and smoking) (Rothman et al., 1997). A several-fold range of CYP2E1 activity has been observed in many human studies.

Hydroquinone and related hydroxylated metabolites can be converted to benzoquinones by bone marrow myeloperoxidase. This is countered by the activity of NQO1 (NAD(P)H:quinone oxidoreductase), which converts the toxic quinones back to hydroxybenzenes. In the same case-control study of BZ poisoning cited above (Rothman et al., 1997), subjects were typed for a polymorphism in NQO1 (C → T transversion at codon 609). Subjects homozygous for this loss-of-function mutation were over-represented among poisoning cases, yielding an odds ratio of 2.6 (1.1–6.6 95%CL; adjusted for age, sex, BMI, alcohol, and smoking). The risk for BZ poisoning in subjects with both metabolic risk factors (rapid hydroxylators/NQOl-) was estimated to be 7.8-fold higher (1.9–32.5 95%CL; adjusted for age, sex, BMI, alcohol, and smoking) when compared to subjects who were slow chlorzoxazone excretors and wild-type or heterozygous at the NQO1-609 locus. People with this combination of risk factors made up 11% of the control group. Therefore, the distribution of these risk factors in the general population could be an important modulator of leukemia risk. In order to incorporate variability in susceptibility into risk assessment, an estimate of the distribution of the risk factors in the at-risk population is needed.

3.3.11.3. Biomarkers of early effect. Dose–response curves obtained from modeling leukemia data are currently extrapolated below the range of the observable data by applying default assumptions. Surrogate biomarkers that provide relevant effect data at lower doses than are available in BZ-induced leukemia datasets could supplement the low-dose extrapolation process. Biomarkers of rate-limiting events on the leukemogenesis pathway would be the most relevant for this purpose. For risk assessment applications, measures of specific chromosomal lesions, both structural and numerical, in bone marrow stem cells from human subjects with well-documented exposure levels would perhaps be ideal biomarkers, as suggested above. However, such an ideal dataset is not available and analysis must therefore be limited to biomarkers with less specificity for exposure and less certain link to leukemogenesis.

(a) *Non-genotoxic effects.* Dose–response modeling of peripheral white blood cell counts is one option for supplementing the modeling of leukemia data. As discussed above, overt hematotoxicity is associated with an elevated risk of leukemia in BZ-exposed patients. There are data available from several exposed cohorts (cited in Section 3.3.5), which could be the subject of a quantitative analysis. Disadvantages of this biomarker for risk assessment purposes can be noted. First, while there is good evidence that pancytopenia is a risk factor and has been associated with high-dose cases of leukemia, a link between mild cytopenia and risk of leukemia has not been established. In addition, there are few cohorts with low exposure ranges for which peripheral cell counts are available, and at least one such study did not find an effect (Collins et al., 1997). Further, one mechanism that has been proposed for lymphocytopenia acts on interleukin 2 response (Li et al., 1997). The dose–response may be specific to T-cells and thus not extrapolable to bone marrow damage.

(b) *Genotoxic effects.* Biomarkers of genetic damage that have been assessed in the peripheral blood of exposed individuals include micronuclei (MN), sister chromatid exchange (SCE), and chromosome aberrations (CA). In addition, loss of heterozygous expression of glycophorin A on the surface of erythrocytes has been determined in blood samples from a small exposed cohort. These potential markers are discussed further below.

Micronucleus. BZ and its metabolites induce micronuclei (MN) in experimental animals and in cultured cells by both chromosome lagging and breakage. Thus, MN could potentially be an appropriate indicator of the most relevant forms of BZ-induced chromosomal damage. However, MN studies in occupational cohorts have not reported consistent associations of the marker with BZ exposure (Pitarque et al., 1996; Hogstedt et al., 1991; Surralles et al., 1997; Tompa et al., 1994).

Sister chromatid exchanges. SCEs have been measured in BZ-exposed persons. However, there are mixed conclusions about the utility of SCEs as an exposure biomarker. In some studies, SCE is correlated with exposure, while in others the association is weak or absent. More importantly, the relevance of SCE as a biomarker of effect is not clear. Without clear rationale to link this marker to leukemogenesis, SCEs are not likely to be useful as a non-tumor surrogate for dose–response assessment for BZ.

Glycophorin A (GPA) variants. The GPA assay identifies the products of two alternative GPA alleles. GPA is expressed on the surface of peripheral erythrocytes, but is produced before the cell loses its nucleus. Mutations expressed by these cells must occur in precursor erythroid cells or stem cells in the bone marrow.

The assay detects a spectrum of mutational mechanisms, but the most significant increase in a group of exposed workers (Rothman et al., 1995) were changes that arose through gene conversion, such that heterozygous individuals became homozygous for one of their two alleles. Thus, GPA is relevant, increased in exposed workers, and most importantly, assesses damage that occurs in the target tissue. However, there is currently a single dataset from a small number of workers, and only summary statistics from exposure groups were reported. If applied to more exposed groups, GPA could prove a useful quantitative indicator of BZ's genotoxicity but only at high levels of exposure.

Structural chromosome aberrations. As discussed above, structural CA are involved in secondary leukemias and are likely to be an early event in the process of leukemogenesis. In contrast to MN and SCE results, the CA response has been more consistently associated with exposure. Numerous studies have reported elevated levels of CA in lymphocytes of workers exposed to BZ when compared to reference groups (reviewed in Zhang et al., 2002).

There is now evidence from two large prospective studies which indicates predictive value for cancer risk of structural CA in peripheral blood lymphocytes (CA-PBL). Bonassi and colleagues (Bonassi et al., 1995) collected data from a number of studies which together assessed CA-PBL in 1455 Italian subjects. The subjects were categorized into high, medium, and low frequency tertiles of CA-PBL and followed up for cause of mortality. A significantly elevated SMR was seen for all cancers in the high and medium groups compared to the low frequency group. In the high CA tertile, an SMR of 182 was observed (based on 20 cancer deaths versus 11 expected). The SMR for lymphatic/hematopoietic neoplasms increased with CA level: zero, one, and four deaths for these causes were observed in the low, medium, and high frequency groups, respectively. The results support the ability of CA-PBL to predict cancer risk in humans, and, although the number of cases is small, a quantitative relationship with risk of hematopoietic cancers has been suggested.

The results of the Nordic Study group cohort (Hagmar et al., 1994) agree with those of Bonassi et al. The incidence of all cancers was assessed for a cohort of 1984 subjects who had been previously evaluated for CA-PBL. Increased cancer incidence was observed in the tertile with the highest level of CA. The point estimate of the incidence ratio, standardized to incidence rates for the four countries participating in the study, was 2.1 (39 cases versus 19 expected). There were no incident leukemia cases at the time of the most recent published follow-up; thus, the association between CA frequency and leukemia cannot yet be evaluated for this cohort. Results from these two large cohorts, and the mechanistic data discussed above together lend credence to the use of CA-PBL as a marker of early biological effect that may predict leukemia risk in BZ-exposed humans.

3.3.12. Summary and conclusion

In summary, there are numerous mechanisms by which BZ and/or its metabolites are able to affect bone marrow cells and/or their microenvironment. BZ-induced leukemogenesis is almost certainly a complex process with multiple contributing factors and more than one possible mechanism. While the complete process is not yet clear, we and most other investigators agree that the induction of specific chromosomal alterations in hematopoietic stem cells is likely to be one of the critical elements. An ideal biomarker of BZ's early effects would therefore be a measure of leukemia-specific chromosome aberrations in bone marrow progenitor cells. Since it is difficult, and perhaps unethical, to sample bone marrow in human population studies, the next best thing would be data on the association between BZ exposure and leukemia-specific aberrations in peripheral blood cells. As indicated above, only one small dataset is available at present and is not informative with regard to lower levels of exposure. Since classical chromosome aberrations are likely to be predictive of future cancer risk and have been measured in a large number of studies of BZ exposed populations, including those exposed to low levels, we suggest that this data be analyzed to provide additional insight into the shape of the dose–response curve for BZ in the low dose region. A preliminary analysis of the published data has been performed by Fanning, Shang, and Smith but is not yet published.

While these chromosome aberration studies comprise the largest source of data on genetic damage in BZ-exposed humans, some caveats must be placed on results obtained from them. The first concerns the relatively poor exposure assessment components of most of these studies and laboratory variability in scoring methods. Secondly, using structural chromosome aberrations as a surrogate risk marker assumes that the dose–response relationship can be extrapolated to the dose–response for BZ and leukemia. However, we have concluded that there are multiple mechanisms that contribute to BZ-induced leukemia. It is reasonable to suggest that different pathways have different dose-responsiveness. Therefore, the utility of a single biomarker analysis for predicting leukemia risk at low doses is likely to be limited. However, until more ideal resources become available, it is worth extracting what information the data from these studies can provide to the risk assessment process.

4. Principles for the use of non-tumor data

From the working group's discussion of the three case studies and of cancer risk assessment in general, several

observations (principles) emerged that are captured and illustrated in the following paragraphs. Most are simple, perhaps even intuitive observations, but they are offered in the belief that it is sometimes useful to state (or restate) the obvious.

4.1. Non-tumor data is critical for determining mechanism/mode of action

As defined by EPA (U.S. Environmental Protection Agency, 1999), a carcinogenic mode of action consists of those key events and processes starting with the interaction of an agent with a cell, through operational and anatomical changes, that result in cancer formation. Mechanism of action is a more complete and detailed molecular description of events. Examples of non-tumor information involved in establishing a mode of action are nature of metabolism, information on adducts or receptor binding, inhibition of cell death, promotional effects, mutagenicity or chromosomal effects, immune suppression, and others. Understanding mode of action permits rational extrapolation across species and from high to low doses. EPA guidelines highlight mode of action as a key factor in identifying the most appropriate method for low dose extrapolation.

VC is a compound with a straightforward mode of action. VC is metabolized to a reactive metabolite which binds to DNA, forming DNA adducts that, if not repaired, lead to mutations and tumor formation. VC metabolites are mutagenic in the Salmonella assay and occupational exposure to VC has resulted in chromosome aberrations, micronuclei, and SCE in cells of exposed workers.

BZ provides an example of a compound for which the mode of action is not well understood. BZ is metabolized to reactive metabolites, but which metabolite(s) are critical is not known. Processes such as altered gene expression, cytotoxicity, chromosomal changes, bone marrow toxicity, and cell proliferation are thought to be important. It is believed that there are likely multiple mechanistic pathways leading to leukemogenesis from exposure to BZ (Smith, 1996). Numerous modes of action including genetic mechanisms involving direct and indirect DNA damage as well as epigenetic mechanisms affecting bone marrow cell proliferation, differentiation, and clonal selection may be important. Lack of understanding of the mode of action of BZ makes predicting risk from BZ exposure difficult.

BD represents a chemical with strong species differences in carcinogenicity and metabolism. As such, it presents vast problems in risk assessment. The use of non-tumor data should be most helpful in understanding critical differences that need to be considered. Unfortunately, there remain some critical gaps in our knowledge. However, thorough reviews of the data assist in pointing out important questions that still need answering. Additional biomarker data that are specific for DEB and HMVK are needed. Likewise, biomarkers can shed light on whether or not important gender differences exist in humans. By critically analyzing the data and the issues, those studies that are needed to enhance the scientific basis of human risk assessment can be identified and pursued in the near future.

4.2. The more we understand about the applied dose → metabolism → adduct → mutation → effect continuum, the more confidence we can have in a cancer risk assessment

The validity of any risk assessment scheme is crucially dependent on the quality of the biological data used in its development. In general, to be of use in a predictive model, non-tumor data must fit into an established mechanistic picture of how the compound produces cancer.

Non-tumor data represent observable properties of the toxicological process that can be used in four general ways: (1) to better understand the chain of events linking exposure to effect, (2) to provide an internal measure of exposure, (3) to identify internal changes predictive of final effect, and (4) to assess the underlying susceptibility of an organism. The extent to which non-tumor data is useful in risk assessment depends on its connection to the causal pathway leading from exposure to effect, and on the level of knowledge concerning the consequences of changes in the biomarker in terms of toxicological endpoints (Benford et al., 1998). The more complete this picture, the more confidence one has in risk predictions based on non-tumor data.

4.3. Non-tumor data is an important component of a weight-of-evidence approach for classification of a compound as a human carcinogen

When classifying an agent as a human carcinogen, EPA guidelines call for a weight-of-evidence approach, integrating all relevant human, animal, and mechanistic data into a coherent picture of a compound's carcinogenic potential in humans. The weight-of-evidence approach maximizes input of scientific knowledge regarding animal carcinogenicity, the mechanism of action in animals, similarity (or lack of similarity) of the process in humans, and confirming information from epidemiologic studies. Non-tumor data play a vital role in this process.

Classification of a compound as a human carcinogen is straightforward when there is strong evidence of carcinogenicity from epidemiologic studies. VC is an example of the straightforward approach. EPA used a combination of tumor and non-tumor data to classify vinyl chloride as a human carcinogen. The weight of evidence for VC human carcinogenicity is based on: (1) strong epidemiologic evidence of a causal association between occupational exposure to VC and the develop-

ment of angiosarcoma; (2) reproducible evidence of carcinogenicity in rats, mice, and hamsters by both the oral and inhalation routes; (3) mutagenicity and DNA adduct formation by VC and its metabolites in numerous in vivo and in vitro test systems; and (4) a clear understanding of mode of action in both animals and humans.

Dioxin provides an example at the other end of the spectrum. Despite multiple human studies, no strong evidence for the carcinogenicity of TCDD in humans has emerged. TCDD has been shown to be a strong multi-site and multi-sex animal carcinogen, much is known about the mechanism of action, and there is strong evidence that TCDD's mode of action is the same in humans as in animals. Based on this information, EPA has classified dioxin as a human carcinogen.

EPA believes that non-tumor data can be used to establish human carcinogenic potential when, in the absence of conclusive epidemiological evidence, there is compelling evidence of carcinogenicity in animals and mechanistic information in animals and humans demonstrates similar modes of carcinogenic action.

4.4. Non-tumor data can improve the validity of extrapolating from high doses to low doses.

Morinello et al. (2002a) measured the formation and repair of DNA adducts in rats exposed to 0, 10, 100, or 1100 ppm VC for five days or four weeks. Due to saturation of metabolic activation, the number of adducts increased in a supralinear manner. Exposure to 10 ppm vinyl chloride for five days caused a 2–3-fold increase in etheno Gua adducts over that of controls, while four weeks' exposure resulted in a 5-fold increase. Exposure to 100 or 1100 ppm VC for four weeks caused a 37-fold increase in adduct levels. These data indicate non-linearity in the dose–response function, and are consistent with saturability in VC metabolic activation.

4.5. Non-tumor data can provide a basis for extrapolating risk between species and between routes of administration

Human risk assessment has to deal with issues of interspecies extrapolation, high-to-low dose extrapolation, and individual susceptibility. The use of non-tumor data can shed considerable light on these issues. The use of either tissue concentration of reactive metabolite or DNA adduct levels to provide a reliable measure of internal dose to target tissue can decrease uncertainty in both interspecies and dose route extrapolation. When extrapolating data between species, doses, tissues, and endpoints should be comparable. Knowledge of the toxicokinetics of the agent is required in order to determine the most appropriate metabolite, tissue, and sampling times, and the relevance of the results obtained. This principle points to the need for low dose chronic exposure animal studies to obtain biomarker endpoints for comparison with biomarker endpoints obtained in human studies.

VC is a known human carcinogen via the inhalation route. It is not know to be a carcinogen via the dermal absorption route. However, EPA classified VC as highly likely to be carcinogenic by the dermal route based on knowledge of its mode of action and the fact that VC is absorbed via skin and distributed throughout the body.

Experimental animal data also demonstrate that BZ is metabolized to the same products whether it is inhaled or ingested. Therefore, it is reasonable to extrapolate from inhalation dose–response to estimate an equivalent oral dose–response.

4.6. Pharmacokinetic models reduce uncertainty in extrapolating from animals to humans

Physiologically based pharmacokinetic (PBPK) models are based on the anatomy, physiology, and biochemistry of an organism and can be used to generate chemical concentration time-course predictions for specific tissues and organs. Such models aid in understanding the relationship between applied dose and dose to target tissue. Pharmacokinetic models enhance the risk assessment process in three major ways: (1) provide a measure of internal dose to target tissue that can be used as a basis for interspecies extrapolation; (2) aid in extrapolation between routes of administration that allows for expanded use of animal bioassay data; and (3) determine the relative importance of different metabolic pathways.

VC provides an example of the use of a pharmacokinetic model to derive risk estimates. Although human cancer epidemiologic studies are available for VC, exposure data from most of these studies are inadequate to derive risk estimates. Thus, EPA used a pharmacokinetic model along with animal tumor data to estimate the human risk of VC exposure. After accounting for pharmacokinetic differences, the model predicted that rats have a greater steady-state concentration of the active metabolite of VC than humans and thereby greater risk. The use of a pharmacokinetic model resulted in an inhalation cancer risk lower than that calculated without its use. The pharmacokinetic model was also used to perform route-to-route extrapolation of the doses used in the oral animal cancer study.

4.7. DNA adduct data can be useful in risk assessment

DNA adducts are produced by the reaction of compounds or their metabolites with DNA. They are of interest for two reasons: they provide biomarkers of exposure that can be used in low dose extrapolation and they are predictive of genetic damage. As a biomarker, DNA adducts provide a measure of exposure that integrates absorption, distribution, metabolic activation/

detoxification, and DNA formation and repair. DNA data also provide a marker of the biologically active fraction of dose. Uses of DNA adducts in risk assessment range from providing information on low dose extrapolation to aiding in the interpretation of epidemiologic studies.

An example from VC illustrates this last use. Early epidemiologic studies suggested that VC caused brain tumors, while more recent, larger studies do not. Morinello et al. (2002b) have shown that while large amounts of VC adducts form in the livers of exposed animals, only endogenous amounts are found in the brain. Thus, DNA adduct data do not support a causal relationship between VC and the induction of brain cancer in occupationally exposed workers.

DNA adducts can also provide a biomarker of exposure. Tissue concentrations of certain environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), cannot be analyzed directly because the parent compound is rapidly metabolized. These metabolites and their DNA adducts, however, accumulate to high levels in certain tissues or body fluids, thus allowing detection of exposure and indication of potential harm.

4.8. Biomarkers of genotoxicity increase confidence in a proposed approach to cancer risk assessment

BD illustrates how biomarkers of genotoxicity can be qualitative indicators of carcinogenicity in a species. BD is carcinogenic through its electrophilic metabolites. These, in turn, are genotoxic in all systems tested. The genotoxic potencies of BD metabolites are in the order: butadiene diepoxide (DEB) $\gg$ butadiene monoepoxide (EB) $\gg$ butadiene monoepoxide-diol (EB-diol). Mice and rats differ in their efficiencies in producing the different metabolites, with mice more adept at the oxidative steps resulting especially in DEB and EB. This would predict that mice are also more susceptible to both gene and chromosome level mutations following exposures to BD. This, in fact, is what has been observed, with no increase in chromosome level mutations being observed in rats following exposure to BD. As mice are far more susceptible than rats to the carcinogenicity of BD, the carcinogenicity of BD in a species is apparently related to its genotoxicity. It will be important to document genotoxicity in humans. However, the results of molecular epidemiological studies in humans have been conflicting. It cannot be said with certainty at this time whether BD at the exposures experienced in modern industry does or does not have in vivo genotoxic effects in humans. However, given the database in animals, such non-tumor data will be important qualitative indicators of cancer risk in humans.

The high association of genotoxicity with carcinogenicity in rodent species supports this principle. The parallelism between metabolic profiles, hemoglobin adducts, DNA adducts, and frank genotoxic endpoints (mutations and chromosome aberrations) strongly supports this mechanism of carcinogenicity. (The findings of oncogene and tumor suppressor gene mutations in the tumors of mice exposed to BD adds additional support.)

4.9. Biomarkers must be selected and interpreted in light of what is known about mechanism of action.

To be of use in a predictive model, non-tumor data must fit into an established mechanistic picture of how the compound produces cancer. Biomarkers of effect are more appropriate as indicators of genotoxicity and defining mechanism of action than are biomarkers of exposure. Biomarkers of exposure are more appropriate for defining true internal doses resulting from external exposures, and for identifying metabolic intermediates resulting from the external exposures. Hemoglobin adducts are biomarkers of exposure whereas DNA adducts may be biomarkers of exposure and biomarkers of effect.

This principle was applied in the recent large transitional epidemiological study of BD exposed Czech workers, where urinary metabolites and hemoglobin adduct concentrations documented exposure levels and the production of in vivo electrophilic metabolites. The negative genotoxicity findings (i.e., no *HPRT* mutations or chromosome changes) are all the more convincing because of these positive exposure responses.

4.10. Biomarkers, as in vivo dosimeters, can improve confidence in exposure estimates

This is true only after the specific biomarkers of exposure have been *validated* in terms of the degree to which they truly reflect external exposure levels. To evaluate the use of a biologic measurement as a biomarker, it is necessary to understand the relationship between the marker and the event or condition of interest. Determining the specificity and sensitivity are critical components of the evaluation process. Validation requires a transitional epidemiological study where the external exposure is precisely known (independent variable) and the behavior of the biomarker is assessed (dependent variable).

4.11. Combination of multiple biomarkers may be necessary to interpret data

Biologic markers can act as quantitative measures of chemical exposures and biologically effective doses, as well as early warning signals of biologic effect. However, often a combination of biomarkers must be used to obtain an unambiguous picture of events.

A specific example is the importance of data from biomarkers of exposure to BD (i.e., urinary metabolites and hemoglobin adducts) in interpreting the negative genotoxicity data (mutations and chromosome changes) in the study of BD exposed Czech workers. Unfortunately, there is still a need for additional biomarker data for DEB and HMVK. When these data become available, it should be possible to greatly improve our quantitative assessment of metabolism and carcinogenesis across the three species that have been studied in detail so that the best scientific data are used to estimate human risk.

BZ provides another example. The ideal biomarker of effect for BZ would be a measure of leukemia-specific chromosome aberrations in bone marrow progenitor cells. Since it is difficult to sample bone marrow in human population studies, the next best source would be data on leukemia-specific aberrations in peripheral blood cells. This biomarker is also difficult to evaluate. Thus, the classical analysis of structural chromosomal aberrations, which have been measured in a large number of studies of BZ exposed populations and at low exposure levels, are the next best choice. However, using structural chromosome aberrations as a surrogate risk marker assumes that the dose–response relationship can be extrapolated to the dose–response for benzene and leukemia. Since, there appear to be multiple mechanisms that contribute to BZ-induced leukemia, it is reasonable to suggest that different pathways have different dose-responsiveness. Therefore, the utility of a single biomarker analysis for predicting leukemia risk at low doses is likely to be limited.

4.12. Endogenous levels of biomarkers can provide information for risk assessment

Endogenous levels of DNA and hemoglobin adducts have consistently been detected in tissues obtained from unexposed animals and humans. These background adducts are formed through a variety of natural processes such as lipid peroxidation and in some cases are identical to adducts formed as a result of chemical exposure. For example, Swenberg has shown that lipid peroxidation and exposure to VC produce identical DNA adducts. Data from rats suggest that typical human exposures to VC (0.1 ppm) may be associated with a 5% increase over the endogenous levels.

Another example of endogenous adducts is the natural production of ethylene oxide-related DNA and hemoglobin adducts resulting from oxidation of ethylene (a natural biological product) to ethylene oxide via lipid peroxidation (Fennell, 1996). The body burden of ethylene oxide in human tissues derived from endogenous ethylene production is estimated to be 0.17 pmol/ml tissue (Filser et al., 1992). An exposure to 15 ppb atmospheric ethylene in the work environment results in an additional tissue concentration of 0.08 pmol/ml tissue, a number consistent with hemoglobin adduct measurements.

Endogenous adducts set a lower bound on the usefulness of DNA adducts as a guide to shape of the dose–response at low dose. For VC, background DNA adduct levels occur at a VC equivalent exposure of about 0.1 ppm or a lifetime cancer risk of 1×10^{-3}. Thus, DNA adducts resulting from VC exposure can only provide information on the shape of the dose–response for about two orders of magnitude below the doses used in the animal bioassays.

High background levels often suggest *non-specificity* of the biomarker. Such levels must be interpreted in light of other more specific biomarker responses included in the same study. For example, high *HPRT* variant frequencies were observed in control workers in the recent study of BD exposed Czech workers. The negative response of the more specific biomarkers of BD exposure (i.e., urine metabolites, some hemoglobin adducts) in these same controls in the same study insured that the high mutation backgrounds were *not* due to BD.

4.13. The metric used for a biomarker must be appropriate to the endpoint

This can be amplified to say that all biomarkers have their own characteristic expression (or manifestation) times following exposures and their own characteristic persistence intervals, after which they decay. Acute exposures must take these characteristics into consideration for each biomarker, although they are not as important for chronic stable exposures.

VC illustrates this point. VC metabolites form four distinct adducts with DNA. The oxoethyl adduct is the major liver DNA adduct, representing 98% of all adducts. However, it is non-mutagenic when tested in in vitro mutational assays. The less common etheno adducts have been shown to cause the same mutations in the K-ras gene as observed in VC-induced human tumors and are the likely cause of VC-induced carcinogenesis. Thus, indiscriminant use of DNA adducts, as predictors of genetic damage may be misleading.

4.14. For a given endpoint, in vivo data are stronger than in vitro data and human data are stronger than animal data

In vitro data bypass considerations of in vivo metabolism. This critical factor in risk assessment, therefore, cannot be assessed. Human data are stronger than animal data because they are ultimately the most relevant. However, it must be remembered that humans are highly outbred and heterogeneous for all susceptibility and resistance factors.

4.15. Biomarkers provide important indications of susceptibility

Non-tumor data can offer insights into the reasons why people respond to stressors differently. For example, polymorphisms affecting metabolism can be an important contributor to susceptibility. Researchers (Skibola et al., 1999) report a lower risk of leukemia if certain genetic variants of an enzyme involved in folate metabolism are present. They speculate that the less risky alleles shift the metabolic balance to allow better copying of DNA, thus avoiding errors that lead to cancer. Studies in humans exposed to BZ have identified an enzyme polymorphism involved in BZ metabolism that confers human susceptibility to BZ-related disease (Rothman et al., 1996). The 60-fold variation in human microsomal potential to metabolize BD (Seaton et al., 1995) suggests a large variation in individual susceptibility. However, when in vivo data on hemoglobin adducts are used, a 95% confidence limit suggests that the variation is more like 10-fold (Swenberg et al., 2001). For vinyl chloride, the observation that the concentration of VC adducts measured in weanlings is generally two to three times greater than in adults (under the same exposure conditions) supports EPA's use of a 2-fold safety factor for children in its risk assessment (U.S. EPA, 2000). While data on CYP2E1 expression in children have not been extensively examined yet, this too can be done to utilize the best science in risk assessments. These studies illustrate the significant role that non-tumor data can have in defining susceptibility to carcinogen exposure.

5. Conclusion

There is a growing recognition that non-tumor data can play an important part in cancer risk assessment. Non-tumor data include information on metabolism, formation of DNA adducts, various types of genetic damage, pharmacokinetic models, information on mode of action, etc. However, to date few of these data have actually made their way into risk assessments. To highlight the types of non-tumor data currently available and promote their increased use in risk assessment, this paper reviews non-tumor data for three widely studied chemical compounds: 1,3-butadiene, vinyl chloride, and benzene. These compounds were selected because of the varied array of non-tumor data available and because they have both animal and human data. The major use of non-tumor data in cancer risk assessment to date has been to establish the mode of action by which toxicity is induced. Better understanding of the mode of action is a critical first step in improving the cancer risk assessment process. This involves more than just knowing that a chemical, for example, is metabolized, forms DNA adducts that cause mutations, which ultimately can lead to cancer. The BD example shows that DNA and hemoglobin adduct data can help identify which metabolite is responsible for adduct formation. The VC example shows that total DNA adduct levels can be a misleading dosimeter and that a minor adduct can be the major contributor to cancer initiation.

Cancer risk assessment often requires extrapolation of the dose–response relationship below the dose range of the tumor incidence data. Non-tumor data on precursor effects may be combined with the tumor data to extend the dose–response below the tumor data. In such a process, improved knowledge of pharmacokinetics, cell proliferation, DNA and hemoglobin adducts, and mutation formation are useful. The VC example demonstrates that DNA adduct data can be used to extend the dose–response curve below the dose range of the animal tumor data and to increase understanding of its shape. VC also illustrates the importance of endogenous adducts. Since these endogenous adducts are identical to those derived from VC, the endogenous levels can be useful in determining vinyl chloride exposures that are not likely to result in increased cancer rates in humans.

Increased precision in extrapolation of animal cancer bioassay data to humans is another major use of non-tumor data. Applications of such data include adjustment of dose for interspecies differences in body mass, metabolic rates and pathways, and uptake, distribution and excretion. The VC example demonstrates the value of PBPK models in cancer risk assessment. BD demonstrates comparisons using DNA adduct levels and mutation rates in mice, rats, and humans as a means of determining the relative sensitivity of these species. The absence of N^2,3-eGua adducts in the brain of rats exposed to VC and comparison of N^2,3-eGua adducts in young and adult rats provided important information supportive of EPA's risk estimation for vinyl chloride.

We concluded this document with 15 principles that should prove helpful in applying non-tumor data in cancer risk assessment. Much work remains to be done. But as the three chemicals considered in this paper demonstrate, non-tumor data are already making critical contributions to more realistic risk assessments. We predict further significant progress in the future.

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