



Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment

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There is a strong need for science-based risk assessment that utilizes known data from diverse sources to arrive at accurate assessments of human health risk. Such assessments will protect the public health without mandating unreasonable regulation. This paper utilizes 30 years of research on three “known human carcinogens”: formaldehyde, vinyl chloride (VC), and ethylene oxide (EO), each of which forms DNA adducts identical to endogenous DNA adducts in all individuals. It outlines quantitative data on endogenous adducts, mutagenicity, and relationships between endogenous and exogenous adducts. Formaldehyde has the richest data set, with quantitative data on endogenous and exogenous DNA adducts from the same samples. The review elaborates on how such data can be used to inform the current risk assessment on formaldehyde, including both the biological plausibility and accuracy of projected risks. Finally, it extends the thought process to VC, EO, and additional areas of potential research, pointing out needs, nuances, and potential paths forward to improved understanding that will lead to strong science-based risk assessment.

Key Words: DNA adducts; endogenous; exogenous; carcinogenesis; epidemiology; risk assessment.

DNA adducts represent key events in mutagenesis and carcinogenesis. For many years, they were thought to be solely the product of exposure to chemical carcinogens. In the past 25 years, however, select DNA adducts were also identified in cellular DNA from tissues of animals and humans not known to be exposed to carcinogens. A major pathway for the formation of such endogenous DNA adducts has been shown to be oxidative stress (Ames, 1989; Dedon, 2008; Dedon *et al.*, 1998; Lunec, 1998; Marnett, 2000; Swenberg *et al.*, 2008). However, oxidative stress is not the only cause of endogenous DNA damage, as formaldehyde and acetaldehyde also induce DNA

adducts. The most common endogenous lesions found in DNA are the aldehydic lesions associated with apurinic/aprimidinic sites that arise from the loss of DNA bases because of oxidative stress and chemical depurination of alkylated and normal bases (Lindahl, 1993; Lindahl and Andersson, 1972; Nakamura *et al.*, 1998, 2000; Nakamura and Swenberg, 1999). Many of the endogenous DNA lesions can result in mutations if DNA replication takes place before they are repaired. Considerable evidence is now accumulating on the numbers of endogenous DNA adducts that are normally present in human and animal cells. Highly sensitive assays are providing accurate data on the quantity of many endogenous adducts. In our laboratory, we utilize Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) and slot blot analyses, taking great care to avoid artifacts. Table 1 illustrates the top 10 endogenous lesions that are measured routinely in our laboratory. These data are presented as “DNA lesions per cell” in order to emphasize the point that even without exogenous exposure to carcinogens, our DNA is far from pristine. This collection of data is most certainly an underestimate of the total number of endogenous DNA lesions, as there are many adducts that we have not examined. It is very likely that every cell has a steady-state background of at least 50,000 endogenous DNA lesions. Under conditions of oxidative stress, this number is expected to increase. In addition to this expanded knowledge on endogenous DNA adducts, the field has greatly benefited from advances in analytical instrumentation (Singh and Farmer, 2006). Just in the last decade, LC-MS/MS instruments have increased detection limits by 10- to 1000-fold. These increases were brought about by the combination of improvements from several technologies, including mass spectrometry (MS) instrumentation with improved ion transmission and detection capabilities, the introduction of ultraperformance liquid chromatography (UPLC) (Churchwell *et al.*, 2005), and the use of

TABLE 1
Steady-State Amounts of Selected endogenous DNA Damage

Endogenous DNA lesions	Number per cell
Abasic sites	30,000
N7-(2-Hydroxyethyl)guanine (7HEG)	3000
8-Hydroxyguanine	2400
7-(2-Oxoethyl)guanine	1500
Formaldehyde adducts	960
Acrolein-deoxyguanosine	120
Malondialdehyde-deoxyguanosine	60
N ² ,3-Ethenoguanine (εG)	36
1,N ² -Ethenodeoxyguanosine (1,N ² -εdG)	30
1,N ⁶ -Ethenodeoxyadenosine (1,N ⁶ -εdA)	12
Total	38,118

capillary-LC, nano-LC, and chip systems allowing for lower mobile-phase flow rates and improved electrospray ionization efficiency (Koster and Verpoorte, 2007; Sikanen *et al.*, 2010). Finally, as stressed in this paper, the use of stable isotope exposures has permitted new approaches that allow distinguishing endogenous and exogenous DNA adducts.

With such florid amounts of DNA damage always present, we expect this background damage to result in “spontaneous” mutations and to be causally involved in the induction of cancer (Ames, 1989; Swenberg *et al.*, 2008). Whereas exogenous DNA adducts are expected to have a dose-response that declines linearly toward zero when exposures are below those that saturate the metabolic activation of procarcinogens, deplete detoxication, or saturate DNA repair pathways. In contrast, mutations always have a quantifiable nonzero background level. There are critical differences between “biomarkers of exposure,” i.e., DNA adducts, that are repairable and “biomarkers of effect,” i.e., mutations, that are heritable. We and others have proposed that these background mutations represent the biology expected to arise from endogenous DNA damage (Jarabek *et al.*, 2009; Loeb and Harris, 2008; Swenberg *et al.*, 2008). This has important implications for epidemiology and quantitative risk assessment. Cancer is a disease of mutations, so an understanding of mutagenic responses at low exposures represents critical information for establishing causality and estimating cancer risk.

In 2005, Dr Chris Wild brought forward the concept of the “exposome,” highlighting the importance of greater understanding of human exposure and emphasizing the need to not only focus on traditional “Omics” in the pursuit of disease reduction (Wild, 2005). This concept has since been expanded in an editorial by Smith and Rappaport (2009) and a meeting at the National Academy of Science held in 2010 (NAS, 2010). The exposome encompasses life course environmental exposures, including lifestyle factors, from the prenatal period onward. It represents a comprehensive measurement of all exposure events, both exogenous and endogenous, from

conception to death. This includes electrophilic molecules that are generated in living cells and organs through normal physiology, lifestyle, and nutrition. The role of endogenous electrophiles in causing Biomarkers of Exposure and Effect in disease causation, as previously reviewed (Jarabek *et al.*, 2009; Swenberg *et al.*, 2008), is consistent with this concept.

Although epidemiology has clearly made many seminal discoveries related to causes of human cancer, there has been a recent call for “epistemological modesty” in the field to reduce the likelihood of false-positive results (Boffetta *et al.*, 2008). Likewise, the recent publication of Science and Decisions (NRC, 2009) suggests that understanding and using Mode of Action in science-based risk assessment is too complicated and concluded that one should simplify cancer and noncancer risk assessments by only considering linear assessments of risk. As the Society of Toxicology celebrates its 50th anniversary, simplifying or ignoring the science, rather than embracing its complexity, fails to make use of our increasing knowledge of biology, genetics, genomics, and metabolomics, and it certainly does not embrace the plea to consider the exposome.

This paper focuses on three chemicals that the International Agency for Research on Cancer (IARC) has classified as “known human carcinogens”: formaldehyde, vinyl chloride (VC), and ethylene oxide (EO). All three induce exogenous DNA adducts that are identical chemically to endogenously formed DNA adducts. Thus, DNA adducts that have the same structure and mutational efficiency as those formed from exposure to these three chemicals are always present in DNA, even when there has been no environmental or occupational exposure. MS analysis allows the use of stable isotope exposures or radioisotope exposures combined with chromatographic separation and accelerator MS (AMS) to compare and contrast the number of identical endogenous and exogenous adducts present under various exposure scenarios. Thus, it is now possible to determine how much of the molecular dose that is present in DNA came from the exogenous exposure and how much was induced by normal biochemistry in the absence of environmental or occupational exposure. Gaining a better appreciation for these relationships will clearly lead to strengthened evidence for or against causality and more effective use of scientific information in chemical-specific quantitative risk assessments. In both epidemiology and risk assessment, enhanced understanding of endogenous and exogenous DNA adducts can be used to place bounds on the plausibility and potency of exogenous exposures to induce disease.

This paper presents an overview of evidence for the existence of specific endogenous DNA adducts of formaldehyde, VC, and EO, followed by sections on sources of endogenous DNA adducts, amounts of DNA adducts, and a short discussion of the mutagenic properties of various DNA adducts when available. This is followed by recent comparisons of endogenous and exogenous DNA adduct numbers associated with exposure to labeled formaldehyde, VC, or EO.

Data are now available for all three of the known human carcinogens in laboratory animals and humans, so comparisons of exogenous and endogenous adducts can be made with reasonable accuracy. Data on formaldehyde are clearly the most robust. We summarize recent findings and then examine its application to proposed risks using new methods to determine how molecular dosimetry information on exogenous and endogenous DNA adducts can influence low-dose risk estimates. We then discuss these issues in relation to the biological plausibility of cancer causation and the low-dose linear risk assumption. Similar approaches are briefly discussed for VC and EO, as those databases are less robust. Finally, we address gaps in our knowledge, potential future research, and implications of this approach for current issues.

FORMALDEHYDE

Formaldehyde is classified as a known human and animal carcinogen according to the IARC (IARC, 2006), causing nasopharyngeal cancer (NPC) in humans and squamous cell carcinomas in the nasal passages of rats (Kerns *et al.*, 1983; Monticello *et al.*, 1996; Swenberg *et al.*, 1980). Additionally, limited epidemiological evidence for the induction of leukemia (LEU) in humans is available (Beane Freeman *et al.*, 2009; Coggon *et al.*, 2003; Hauptmann *et al.*, 2003, 2004, 2009; Marsh *et al.*, 2010). However, whether or not formaldehyde causes LEU remains debatable, as experimental data do not support the induction of LEU (Lu *et al.*, 2010a). As a high-volume industrial chemical, formaldehyde can enter the body through a variety of environmental exposures. Of equal importance, formaldehyde is also an essential metabolic intermediate generated endogenously from serine, glycine, methionine, and choline and also produced from metabolism of xenobiotic chemicals and proteins by demethylation.

Formaldehyde-DNA Adducts and Mutagenicity

Formaldehyde is a very reactive compound, directly targeting diverse nucleophiles of DNA and protein. Typically, formaldehyde can induce DNA adducts including N²-hydroxymethyl-deoxyguanosine (dG), N⁶-hydroxymethyl-deoxyadenosine (dA), and N⁴-hydroxymethyl-deoxycytosine (dC) *in vitro*. Those DNA adducts are considered to be promutagenic, as the adduction occurs on the amino groups participating in Watson-Crick base pairing. Numerous experiments have demonstrated that formaldehyde exposure induces mutations in a variety of test systems ranging from bacteria to laboratory animals. In *Escherichia coli*, exposure to 4 mmol/l formaldehyde for 1 h induced large insertions (41%), large deletions (18%), and point mutations (41%) in the xanthine guanine phosphoribosyl transferase gene (Crosby *et al.*, 1988). The point mutations were transversions at GC base pairs, as revealed by DNA sequencing. GC → TA transversions were

also found in *E. coli* Lac⁺ WP3104P and in *S. typhimurium* His⁺ TA7005 after formaldehyde exposure (Ohta *et al.*, 1999, 2000). DNA sequencing demonstrated that AT → CG transitions were the predominant point mutations in formaldehyde-induced human lymphoblast TK6 X-linked hypoxanthine guanine phosphoribosyl-transferase (HPRT) mutants (Liber *et al.*, 1989). Formaldehyde exposure did not induce gene mutations in the HPRT locus in Chinese hamster V79 cells, but other genotoxic effects such as DNA-protein cross-links, sister chromatid exchange, and micronuclei were found (Merk and Speit, 1998). In addition, several point mutations have been identified by DNA sequence analysis of *p53* complementary DNA from formaldehyde-induced squamous cell carcinomas in nasal tissues of rats, including ₃₉₆C → A (codon 132), ₃₉₈G → T (codon 133), ₆₃₈G → T (codon 213), ₈₁₂G → A (codon 271), and ₈₄₂G → C (codon 281) (Recio *et al.*, 1992).

Endogenous Formaldehyde-DNA Adducts

As mentioned above, formaldehyde is an essential metabolic intermediate generated in all living cells. The endogenous formaldehyde concentration in human blood is ~100 μM, which induces endogenous formaldehyde-DNA adducts. The number of endogenous N²-hydroxymethyl-dG adducts in rats was measured by LC-MS/MS and found to be ~1–7 adducts/10⁷ dG (Lu *et al.*, 2010a). The number of endogenous N⁶-hydroxymethyl-dA adducts in rats was also quantified and shown to be ~1–3 adducts/10⁷ dA across a range of tissues (Cheng *et al.*, 2008; Lu *et al.*, 2010a). The formation of formaldehyde-DNA adducts in humans has also been demonstrated. The adduct number of endogenous N⁶-hydroxymethyl-dA was determined as ~0.7 adducts/10⁷ adducts in human leukocytes (Wang *et al.*, 2009). We now have additional data on endogenous N²-hydroxymethyl-dG adducts in primates where higher numbers of endogenous adducts have been measured in bone marrow (Moeller *et al.*, 2011).

Relationships of Exogenous/Endogenous Formaldehyde-DNA Adducts

Quantifying the number of adducts caused by exposure to formaldehyde has proven difficult because it is confounded by the presence of a substantial natural background of formaldehyde adducts. Recently, our laboratory has employed [¹³CD₂]-formaldehyde for exposure, coupled with highly sensitive MS analyses (Lu *et al.*, 2010a; Lu *et al.*, 2010b; Moeller *et al.*, 2011). This unique strategy allowed us to distinguish DNA adducts originating from endogenous and exogenous sources of formaldehyde based on the different transitions used in selected reaction monitoring (SRM) mode (Fig. 1). The exogenous DNA adducts have a 3 Da mass increase because of the use of [¹³CD₂]-formaldehyde. Through this approach, we have been able to accurately quantify both endogenous and exogenous adducts induced by formaldehyde. The first

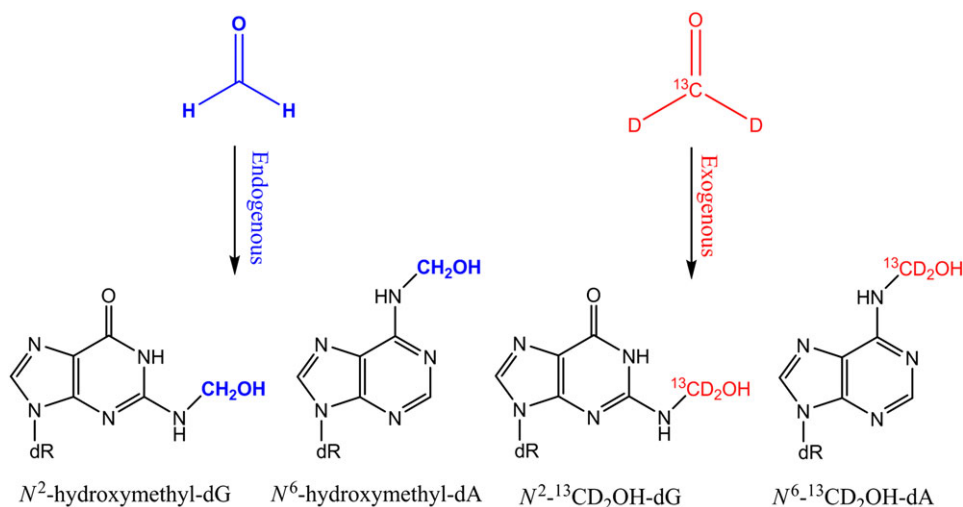


FIG. 1. Formation of hydroxymethyl DNA adducts induced by formaldehyde.

molecular dosimetry study was carried out by exposing rats to 10 ppm [¹³CD₂]-formaldehyde for 1 day (6 h/day) or 5 days (6 h/day) (Lu *et al.*, 2010a). The number of exogenous N²-HO¹³CD₂-dG in 1-day and 5-day nasal DNA samples from rats exposed by inhalation to 10 ppm [¹³CD₂]-formaldehyde was 1.28 ± 0.49 and 2.43 ± 0.78 adducts/10⁷ dG, respectively. The number of endogenous N²-HOCH₂-dG adducts was 2.63 ± 0.73 and 2.84 ± 1.13 adducts/10⁷ dG. In addition, 3.95 ± 0.26 and 3.61 ± 0.95 N⁶-HOCH₂-dA endogenous adducts/10⁷ dA were present, but no exogenous dA adducts were detected. The ratios of exogenous versus endogenous dG adducts were 0.57 ± 0.28 and 1.06 ± 0.40 for the 1-day- and 5-day-exposed groups, respectively. This study also examined sites distant from the point of contact but did not detect exogenous adducts in any distant tissues, such as bone marrow, spleen, thymus, and white blood cells, whereas endogenous dG and dA adducts were present in all tissues analyzed. In order to increase the likelihood of measuring [¹³CD₂]-labeled exogenous adducts in

sites distant to the point of contact, approximately five times more DNA was analyzed.

More recently, we have expanded our studies on molecular dosimetry of formaldehyde-DNA adducts by exposing rats to 0.7, 2, 5.8, 9.1, and 15.2 ppm [¹³CD₂]-formaldehyde for 1 day (6 h/day), which modeled the exposures in a previous cell proliferation and carcinogenicity study (Monticello *et al.*, 1996). Formaldehyde induced nasal squamous cell carcinomas in a highly nonlinear fashion, with no nasal carcinomas at 0.7 and 2 ppm but 1, 22, and 47% carcinomas at 6, 10, and 15 ppm formaldehyde. The exogenous dG adducts were 0.039 ± 0.019, 0.19 ± 0.08, 1.04 ± 0.24, 2.02 ± 0.43, and 11.15 ± 3.01 for 0.7, 2, 5.8, 9.1, and 15.2 ppm exposure, respectively, which clearly illustrated a nonlinear exposure-response, as demonstrated by the fact that 21-fold exposure increase (0.7–15.2 ppm) induced 287-fold higher amounts of exogenous DNA adducts in rat nasal epithelium (Fig. 2). In addition, the ratios of exogenous versus endogenous were determined to be 0.011 ± 0.001, 0.033 ± 0.006, 0.19 ± 0.04, 0.66 ± 0.17, and 2.78 ± 1.08, respectively, for the [¹³CD₂]-formaldehyde exposures, clearly demonstrating that endogenous DNA adducts predominate at low-dose exposures.

Moreover, we have carried out a primate study that exposed monkeys to 1.9 or 6.1 ppm isotope-labeled formaldehyde for 2 days (6 h/day). The exogenous dG adducts in nasal DNA of monkeys after the 2-day exposures were similar to that of rats exposed to 2 ppm for 1 day, whereas the exogenous dG adducts were ~2.5-fold lower in monkeys exposed to 6.1 ppm [¹³CD₂]-formaldehyde for 2 days (6 h/day) compared with rats exposed to 5.8 ppm for 1 day (Fig. 3). These data demonstrated that lower numbers of exogenous DNA adducts were formed in nasal turbinates of nonhuman primates than rats after inhalation exposure to formaldehyde. Moreover, our preliminary data indicate that primates have two- to threefold higher endogenous dG

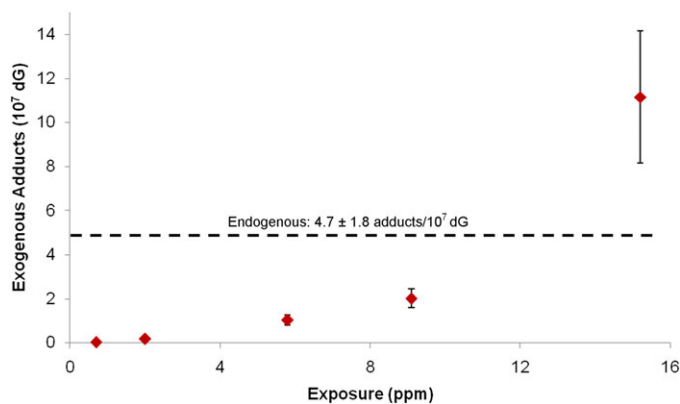


FIG. 2. Molecular dosimetry of N²-hydroxymethyl-dG adducts in rats exposed to formaldehyde.

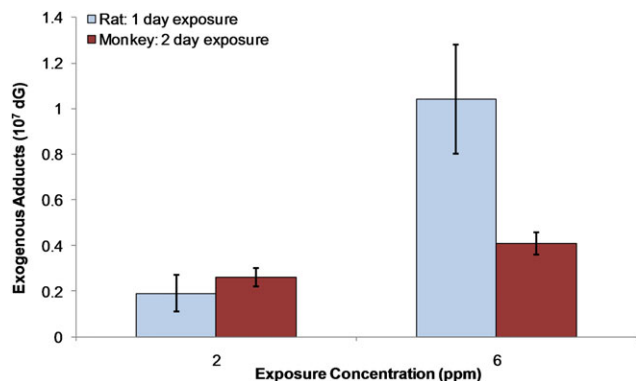


FIG. 3. Formation of exogenous dG adducts in rats and monkeys exposed to 1.9 and 6.1 ppm formaldehyde (1-day exposure for rats and 2-day exposure for monkeys).

adducts in tissues we analyzed. This substantially higher background of endogenous adducts further reduces the ratio of exogenous/endogenous formaldehyde adducts in primates exposed to low concentrations.

Furthermore, we performed a study to determine the half-life of exogenous formaldehyde-DNA adducts. Rats were exposed to 10 ppm [¹³CD₂]-formaldehyde for 1 day (6h) and then sacrificed at 0, 6, 12, 24, 48, or 72 h, respectively. The data showed a rapid loss of nearly half of the adducts during the first 6 h postexposure, followed by a constant rate of loss over the rest of the 72-h experiment (Lu, unpublished data). The initial decrease in [¹³CD₂] adducts is thought to result from cell death because of cytotoxicity, followed by a half-life of ~94 h. When 10-ppm exposures occur daily, this scenario repeats itself. That explains why 5 days of exposure only doubled the number of exogenous adducts.

Application of Data on Endogenous/Exogenous Adducts to Formaldehyde Risk

On 2 June 2010, the U.S. Environmental Protection Agency (U.S. EPA) released its draft Toxicological Review of Formaldehyde—Inhalation Assessment, which reviews the extraordinarily rich database that exists for formaldehyde toxicity and carcinogenicity and describes inferences regarding potential human health risks from environmental formaldehyde exposures that the Agency has drawn from its consideration of this database (U.S. EPA, 2010). In particular, this document sets out a “preferred (plausible upper bound) unit risk estimate” of 0.081 per ppm that is based on adult human data for NPC, Hodgkin lymphoma (HL), and LEU. After application of age-dependent adjustment factors (ADAFs), the overall (plausible upper bound) unit risk estimate that the Agency considers applicable to “full lifetime” formaldehyde exposures is increased further to 0.13 per ppm. In other words, the Agency is suggesting that as many as 13% of people (approximately one in eight) who are exposed continuously to 1 ppm

formaldehyde could develop one or more of these three cancers. The U.S. EPA document also presents the individual unit risk estimates for each of these cancers (0.011/ppm for NPC, 0.017/ppm for HL, and 0.057/ppm for LEU) that were derived by the Agency using (adult) epidemiologic data and cumulative formaldehyde exposure as the dose metric (see Table 6-3, pp 6–41, 6–42 of the draft).

Are these high estimates of extra lifetime human cancer risk credible, even as “plausible” upper bounds? Do they arise as a consequence of real causal associations between workers’ cancer risks and their actual formaldehyde exposures as has been asserted by the Agency? Or, are there other plausible explanations such as small numbers of cancer deaths, residual bias, and uncertainty in the characterization of occupational exposures to formaldehyde and/or other risk factors? We can begin to develop answers to these questions by taking advantage of the newly acquired information regarding specific DNA adducts that arise from the chemical interaction of DNA with formaldehyde of either endogenous or exogenous (inhaled) origin (Lu *et al.*, 2010a; Lu *et al.*, 2010b; Moeller *et al.*, 2011). As has already been noted, these adducts constitute a highly sensitive and specific biomarker of exposure (dose metric) and potential effects (mutations and subsequent cancer development) that is far superior to the necessarily coarse exposure characterizations that have been developed as part of retrospective cohort mortality investigations.

Recent studies employing stable isotope-labeled formaldehyde afford the ability to differentiate between formaldehyde molecules of endogenous and exogenous origin (Lu *et al.* 2010a; Lu *et al.*, 2010b; Moeller *et al.*, 2011). Such research also makes it possible to develop cancer risk estimates with a unique “bottom-up” approach that extrapolates upward from background (endogenous) exposure and response, as opposed to the typical “top-down” approach that often requires downward extrapolation from exogenous exposure levels so extreme as to be potentially irrelevant to the potential risks that might be present at the far lower environmental exposures that are of primary interest. We propose herein a simple empirical model of human cancer risk for conservatively bounding the extra lifetime cancer risk that might arise from low-level formaldehyde exposures, relying upon the dG adduct data as the dose metric and a conservative linear dose-response model. Extra risk estimates obtained with this bottom-up approach can then be compared with those put forward by U.S. EPA in its draft assessment document.

The data of Lu *et al.* (Lu *et al.* 2010a; Lu *et al.*, 2010b; Moeller *et al.*, 2011) for dG monoadducts have been summarized in Table 2. In what follows, we have utilized the dG adduct data for each combination of concentration, number of 6-h exposures, species, and tissue to develop the alternative human cancer risk estimates that are presented in Table 3. The sample size for each dG adduct determination was first used to convert SDs into corresponding SEs as shown in Table 3. The SEs were then used to compute lower 95% confidence bounds

TABLE 2
Formaldehyde-Induced N²-Hydroxymethyl-dG Adducts in Rats Exposed to 10 ppm Formaldehyde for 1 Day or 5 Days, Rats Exposed to Different Concentrations of Formaldehyde for 1 Day, and Monkeys Exposed to 2 and 6 ppm Formaldehyde for 2 Days

Species	Exposure and period	Tissues	N ² -HOCH ₂ -dG (adducts/10 ⁷ dG ± S.D.)	
			Endogenous	Exogenous
Rat	10 ppm/1 day	Nose	2.63 ± 0.73	1.28 ± 0.49
		Bone marrow	1.05 ± 0.14	n.d.
		Blood	1.28 ± 0.38	n.d.
	10 ppm/5 day	Nose	2.84 ± 1.13	2.43 ± 0.78
		Bone marrow	1.17 ± 0.35	n.d.
		Blood	1.10 ± 0.28	n.d.
Rat	0.7 ppm/1 day	Nose	3.62 ± 1.33	0.039 ± 0.011
	2 ppm/1 day	Nose	6.09 ± 3.03	0.19 ± 0.08
	5.8 ppm/1 day	Nose	5.51 ± 1.06	1.04 ± 0.24
	9.1 ppm/1 day	Nose	3.41 ± 0.46	2.03 ± 0.43
	15.2 ppm/1 day	Nose	4.24 ± 0.92	11.15 ± 3.01
		Bone marrow	18.2 ± 0.47	n.d.
Monkey	2 ppm/2 days	Nose	2.49 ± 0.40	0.25 ± 0.04
		Bone marrow	17.5 ± 2.6	n.d.
	6 ppm/2 days	Nose	2.05 ± 0.54	0.41 ± 0.05
		Bone marrow	12.4 ± 3.6	n.d.

Note. n.d., not detected.

on the numbers of “endogenous” dG adducts in nose, bone marrow, and blood (white blood cells).

Because no exogenous dG adducts were detected in any of the distant site tissues, including bone marrow and mononuclear white blood cells, we can state with confidence that if such exogenous adducts were present in these tissues, then their amounts would necessarily have been smaller than the LC-MS/MS-SRM method’s limit of detection (LOD). We have therefore used the method’s LOD (reported as 240 × 10⁻¹⁸ mol, Lu *et al.*, 2010a or 20 × 10⁻¹⁸ mol, Lu *et al.*, 2010b) as a worst-case upper bound on the level of exogenous dG adducts that could be present in bone marrow or mononuclear white blood cells. The above molar LODs were converted to the equivalent 0.0177 or 0.000946 adducts per 10⁷ dG, respectively, using the amount of rat or monkey DNA collected in the distant site tissue samples and the amount of guanine (0.22 for rats and 0.20 for monkeys, expressed as a fraction) that is present in DNA (Casanova *et al.*, 1989, 1991).

Because the endogenous and exogenous dG adducts are chemically indistinguishable following an environmental exposure, we assume that both are implicated to the same extent in the carcinogenicity of low exposures to formaldehyde. The dose-response model that we propose is very simple: a direct, proportional (i.e., linear) relationship between cancer incidence and dG adducts, at least up until local formaldehyde tissue concentrations become so high as to cause cytotoxicity

and related increases in cell proliferation. At exposures of 6 ppm and higher, one would expect to see greater than linear, i.e., nonlinear increases in cancer risk with increasing formaldehyde exposure, and our linear model would need to be elaborated with a multiplicative cell proliferation component to reflect appropriately these high dose-only phenomena.

Our risk model implies that both endogenous and exogenous dG adducts give rise to proportional tissue-specific risks of cancer development. If p_0 represents the background risk of tissue-specific cancer attributable to endogenous formaldehyde-dG adducts and $c_0(\text{dG})$ represents the tissue-specific level of such adducts, then the ratio, $a_0 = p_0/c_0(\text{dG})$, provides an estimate of the unit risk, or potency, of formaldehyde-dG adducts to cause tissue-specific cancer, i.e., it represents the incremental cancer risk attributable to a single adduct per 10⁷ dG.

We do not know the true value of p_0 because we do not know the complete mechanism of cancer development in relation to the formation of formaldehyde-dG adducts in tissues, but we do know that p_0 cannot be any greater than the total background risk of developing a specific cancer (p_0); so in generating our human estimates of cancer risk, we have assumed that all of the total background risks of specific cancers such as NPC, HL, or LEU, are attributable to the level of endogenous formaldehyde-dG adducts in the associated tissues, namely, nose, bone marrow, or mononuclear white blood cells. For NPC, we used the lifetime probability provided in the U.S. EPA draft document, namely, 7.25×10^{-4} (Table C-1, pp. C-3 and Section 5.2.2). For HL (2.3×10^{-3}) and LEU (1.3×10^{-2}), we used the both sexes, all race lifetime risk estimates that are provided in Table 1.14 of SEER Cancer Statistics Review 1975–2007 (Altekruse *et al.*, 2010).

We have also assumed that steady-state exogenous dG adduct levels achieved with a continuous 24 h/day, 7 days/week exposure regimen are related to the amounts measured at each time point in the various adduct experiments as would be predicted by a simple one-compartment kinetic model having a 94-h elimination half-life for exogenous dG adducts (Lu, unpublished data) (mean lifetime = $94/\ln(2) = 135.6$ h). For example, if A_6 represents exogenous dG adducts measured following a single 6-h exposure to a given airborne formaldehyde concentration, A_{s-s} represents the asymptotic steady-state resulting from continuous exposure to the same concentration, and T represents the mean adduct lifetime, then $A_{s-s} = A_6/[1 - \exp(-6/T)] = A_6 \times 23.11$. Similarly, if A_{30} represents exogenous dG adducts measured following two 6-h exposures on consecutive days, then $A_{s-s} = A_{30}/\{[1 - \exp(-6/T)] \times [1 + \exp(-24/T)]\} = A_{30} \times 12.57$. Finally, if A_{102} represents exogenous dG adducts measured following five 6-h exposures on consecutive days, then $A_{s-s} = A_{102}/\{[1 - \exp(-6/T)] \times [1 + \exp(-24/T)] + \exp[-48/T] + \exp[-72/T] + \exp[-96/T]\} = A_{102} \times 6.38$.

To extrapolate from risks estimated at each measured adduct level to those expected at other airborne formaldehyde

TABLE 3

Comparison of Extra Lifetime Risks of Developing NPC, HL, and LEU from Continuous Exposure to 1 ppm Formaldehyde as Estimated Herein Using Formaldehyde-dG Adduct Data (dG-A) from Lu *et al.* (2010a), Lu *et al.* (2010b) and Moeller *et al.* (2011), with Those Estimated by U.S. EPA Using Adult Human Data (Table 6-3, pp. 6–41, 6–42, U.S. EPA draft document)

Cancer type	Background lifetime risk	Endogenous adducts, mean \pm SE (per 10^7 dG)	UCL ₉₅ slope factor, risk per adduct (per 10^7 dG)	Exogenous adducts, mean \pm SE (per 10^7 dG)	dG-A UCL ₉₅ risk estimate at 1 ppm	EPA UCL ₉₅ risk estimate at 1 ppm	Risk ratio, EPA/dG-A
NPC	7.25×10^{-4}	2.84 ± 0.51^a	3.61×10^{-4}	2.43 ± 0.29	1.22×10^{-3a}	1.1×10^{-2}	9.03
		2.63 ± 0.30^b	3.39×10^{-4}	1.28 ± 0.17	2.18×10^{-3b}		5.05
		4.24 ± 0.41^c	2.03×10^{-4}	11.15 ± 1.35	7.49×10^{-3c}		1.47
		3.41 ± 0.21^d	2.36×10^{-4}	2.03 ± 0.19	2.65×10^{-3d}		4.16
		5.51 ± 0.53^e	1.56×10^{-4}	1.04 ± 0.12	1.41×10^{-3e}		7.81
		6.09 ± 1.52^f	2.02×10^{-4}	0.19 ± 0.040	0.96×10^{-3f}		11.4
		3.62 ± 0.77^g	3.08×10^{-4}	0.039 ± 0.011	0.86×10^{-3g}		12.8
		2.05 ± 0.27^h	4.50×10^{-4}	0.41 ± 0.025	0.39×10^{-3h}		28.5
		2.49 ± 0.23^i	3.42×10^{-4}	0.25 ± 0.020	0.54×10^{-3i}		20.5
		HL	2.3×10^{-3}	1.10 ± 0.16^a	2.76×10^{-3}	$< 1.77 \times 10^{-2j}$	$< 6.78 \times 10^{-5j}$
1.29 ± 0.19^b	2.36×10^{-3}			$< 1.77 \times 10^{-2j}$	$< 20.9 \times 10^{-5j}$	> 81.2	
LEU	1.3×10^{-2}	1.17 ± 0.20^a	1.55×10^{-2}	$< 1.77 \times 10^{-2j}$	$< 3.81 \times 10^{-4j}$	5.7×10^{-2}	> 149.4
		1.05 ± 0.081^b	1.42×10^{-2}	$< 1.77 \times 10^{-2j}$	$< 12.6 \times 10^{-4j}$		> 45.2
		12.4 ± 1.82^h	1.38×10^{-3}	$< 1.03 \times 10^{-3k}$	$< 2.96 \times 10^{-6k}$		> 19,256
		17.5 ± 1.31^i	0.85×10^{-3}	$< 1.03 \times 10^{-3k}$	$< 5.47 \times 10^{-6k}$		> 10,420

Note. UCL, Upper Confidence Limit

^aAdduct data for rats after five 6 h exposure to 10 ppm (from Lu *et al.*, 2010a).

^bAdduct data for rats after one 6-h exposure to 10 ppm (from Lu *et al.*, 2010a).

^cAdduct data for rats after one 6-h exposure to 15.2 ppm (from Lu *et al.*, 2010b).

^dAdduct data for rats after one 6-h exposure to 9.1 ppm (from Lu *et al.*, 2010b).

^eAdduct data for rats after one 6-h exposure to 5.8 ppm (from Lu *et al.*, 2010b).

^fAdduct data for rats after one 6-h exposure to 2.0 ppm (from Lu *et al.*, 2010b).

^gAdduct data for rats after one 6-h exposure to 0.7 ppm (from Lu *et al.*, 2010b).

^hAdduct data for monkeys after two 6-h exposure to 6 ppm (from Moeller *et al.*, 2011).

ⁱAdduct data for monkeys after two 6-h exposure to 2 ppm (from Moeller *et al.*, 2011).

^jExogenous dG adducts were not detected in the blood or bone marrow. Upper bound extra risk estimates are based on the LOD in Lu *et al.* (2010a), namely, 0.0177 adducts per 10^7 dG.

^kExogenous dG adducts were not detected in the bone marrow of monkeys exposed to 6 or 2 ppm. Upper bound extra risk estimates for these cases are based on the average LOD in Moeller *et al.* (2011), namely, 0.001034 adducts per 10^7 dG.

concentrations, we have assumed that the relationship between airborne formaldehyde concentration and exogenous dG adducts is linear through zero. Given the nonlinear relationship between dG adducts and airborne concentration that was demonstrated in the dosimetry study of Lu *et al.* (2010b), this assumption will tend to overestimate exogenous adducts at lower concentrations, leading to overestimation of the associated cancer risks.

Finally, we assume that the levels of endogenous and exogenous dG adducts found in various rat and primate tissues are representative of those that would be found in corresponding human tissues after adjusting for species differences in the average flux of inhaled formaldehyde onto surfaces of the respiratory tract. Preliminary results have demonstrated that similar numbers of adducts are formed in rats following a single 6-h exposure and in monkeys exposed to 1.9 ppm for 2 days, thus monkeys get approximately half of the molecular dose that a rat receives. We have used the same rat/human respiratory tract flux ratio (0.46) that U.S. EPA employed in its

multistage Weibull time-to-tumor analysis of rat nasal tumors from the Chemical Industry Institute of Toxicology inhalation bioassays (Section 5.3.6.2 and Table 5-24 in the draft document, U.S. EPA, 2010). For risk estimates derived from the monkey adduct data, we have assumed that the corresponding monkey/human respiratory tract flux ratio is equal to 1.

Table 3 presents the results from using the bottom-up linear extrapolation model and the above-noted assumptions and dG adduct data to calculate upper bound estimates of extra lifetime cancer risk from continuous exposure to 1 ppm formaldehyde. For NPC, our upper bound extra risk estimates span a near 20-fold range from a low of 0.39×10^{-3} (using monkey data for two 6-h exposures to 6.1 ppm) up to 7.49×10^{-3} (using rat data for one 6-h exposure to 15.2 ppm). Despite this broad range, all our NPC risk estimates are smaller than the corresponding U.S. EPA estimate of 1.1×10^{-2} .

Our estimates of HL and LEU risk diverge even more dramatically from the corresponding U.S. EPA estimates. At 1 ppm, our two HL estimates are less than a factor of 3.1

apart from each other but are 81-fold and more than 250-fold, respectively, lower than U.S. EPA's estimate of 1.7×10^{-3} . Our LEU estimates range from 45-fold (using rat data for one 6-h exposure to 10 ppm) to more than 19,000-fold (using monkey data for two 6-h exposures to 6.1 ppm) lower than the U.S. EPA estimate of 0.057. At 1 ppb, our lifetime risk estimates for these two cancers are thus all well below the *de minimus* 1×10^{-6} level. The marked disparities between the adduct-based and epidemiology-based cancer risk estimates for distant sites suggest strongly that the excess risks of HL and NPC that have been reported in association with occupational formaldehyde exposures cannot be due to formaldehyde. If our plausible assumption that formaldehyde-dG adducts provide a valid molecular dosimeter for relating potential human cancer risks to formaldehyde exposure, the far larger risks derived from adult human data are simply not credible.

We are confident that the risk estimates obtained from this simple risk assessment model are conservative for several reasons. Most importantly, this model attributes all the background risk of specific cancers to the endogenous formaldehyde-dG adducts that are found in the corresponding tissues. This background risk automatically includes any contributions to lifetime risk that come from childhood years, so ADAFs do not need to be applied to our risk estimates. Second, we only included the endogenous dG adducts in our risk calculations, even though there are even higher endogenous dA adducts present in the tissues (inclusion of other adducts would further reduce the slope of the assumed linear dose-response relationship between cancer risk and adduct levels). Third, the risk model is linear (and conservative) at low doses because it is, in fact, linear at all doses. Fourth, we used lower 95% confidence bounds on measured endogenous adduct levels to generate corresponding upper 95% confidence bounds on the slope of the model-implied linear relationship between cancer risks and dG adduct levels. Fifth, we have made conservative assumptions on kinetics and dG adduct half-life data in converting adducts measured after a 6 h/day, 5 days/week exposure regimen to those that would be expected from lifetime continuous 24 h/day exposures and in scaling up the respiratory tract deposition of formaldehyde from rats to humans using the same methods that were utilized by the U.S. EPA.

Opportunities exist to replace assumptions that we have made in the absence of information, with data-driven alternatives. For example, our preliminary primate data (Table 2 and Fig. 3) will be expanded to include the distribution of dG adducts in five locations of the nasal passages, trachea, bronchi, and proximal and distal lung, as well as many additional tissues. This will shed light on potential species differences and the effects of oral/nasal breathing versus obligatory nasal breathing in rats in the formation of such adducts. In addition, it should be reasonably easy to obtain data for endogenous formaldehyde adducts in human tissues. We have submitted an Institutional

Review Board protocol to evaluate this. Such data could be used to confirm and/or replace the plausible dosimetric assumption that endogenous formaldehyde-dG adduct levels are similar in rats, monkeys, and humans.

The methodology of Lu *et al.* (Lu *et al.* 2010a; Lu *et al.*, 2010b; Moeller *et al.*, 2011) differentiates clearly between formaldehyde molecules of endogenous and exogenous (inhaled) origin. This remarkable achievement makes it possible to develop cancer risk estimates with a unique bottom-up approach that extrapolates upward from background (endogenous) exposures and responses, as opposed to the typical top-down extrapolation from cancer incidence in laboratory animals subjected to extremely high exposure concentrations. The simple linear exposure-response model we have used to estimate upper bound lifetime human NPC, HL, and LEU risks from inhaled formaldehyde at exposure concentrations of 1 ppm and below capitalizes on this new molecular dosimetry information. Comparison of our risk estimates with corresponding estimates from the U.S. EPA draft document shows the latter to be markedly higher. This discrepancy between results obtained with molecular dosimetry and those that rely on highly uncertain retrospective exposure reconstructions calls into serious question the credibility of attributing increased human mortality from these cancers, particularly at distant sites, to occupational formaldehyde exposures.

For example, endogenous and N^2 - $[^{13}CD_2]$ -hydroxymethyl-dG adducts were readily measured in 20–30 μg of nasal DNA from nonhuman primates; however, no N^2 - $[^{13}CD_2]$ -hydroxymethyl-dG adducts were detectable when 312 μg of bone marrow DNA was analyzed. In contrast, high numbers of endogenous N^2 -hydroxymethyl-dG adducts were present in bone marrow DNA. The number of endogenous adducts in the 312- μg sample of bone marrow DNA was 13.9 adducts/ 10^7 dG. If one constructs a worst-case scenario by assuming that exogenous adducts were just below the LOD in this sample, less than 1 N^2 - $[^{13}CD_2]$ -hydroxymethyl-dG adduct/ 10^{10} dG could have existed. This would mean that less than one exogenous DNA adduct was present for every 13,900 endogenous formaldehyde adducts. It is difficult to conceive of a mechanism by which 1/13,900 identical DNA adducts could drive the biology that leads to carcinogenesis.

In summary, the use of stable isotope formaldehyde exposures has provided new insight and tools for science-based risk assessment. The fact that formaldehyde is present in every living cell cannot be ignored when conducting a cancer risk assessment for inhaled formaldehyde. Until now, we have not known what the relationship was between the number of exogenous DNA adducts derived from the inhaled formaldehyde exposure and the number of endogenous adducts present in bone marrow. Now that the relationship is known, we have to seriously question the biological plausibility that inhaled formaldehyde causes LEU and HL, and start asking much more probing questions about the epidemiology data.

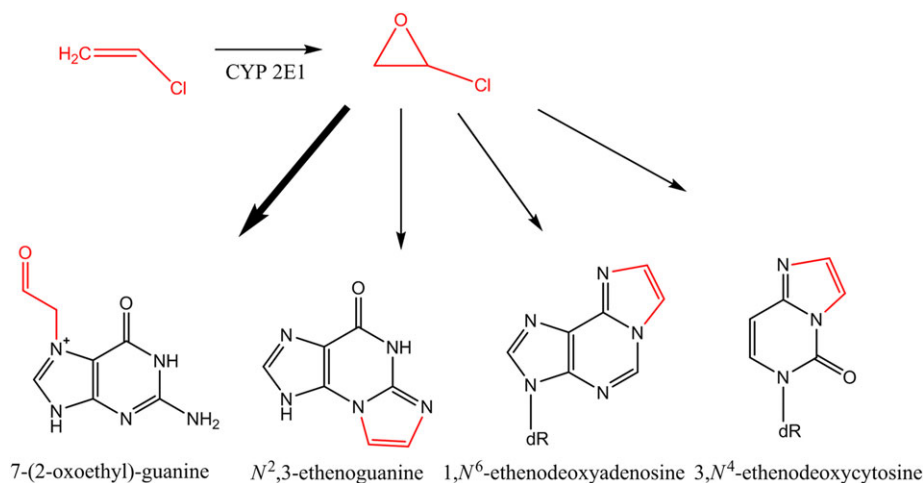


FIG. 4. Metabolism of VC and formation of DNA adducts from CEO.

VINYL CHLORIDE

VC is a known human and animal carcinogen that induces hepatic angiosarcomas (IARC, 1987). The carcinogenic responses for VC are associated with relatively high exposures (≥ 50 ppm). To date, 197 VC workers have developed hepatic angiosarcomas (Kielhorn *et al.*, 2000). All these individuals started work prior to the lowering of the occupational exposure limit to 1 ppm in 1974. Early epidemiology studies also found associations between VC exposure and brain tumors in workers (Cooper, 1981; Waxweiler *et al.*, 1976; Wong *et al.*, 1991). As the epidemiology studies got stronger through larger cohorts, longer follow-up, and exposure was examined more thoroughly, this association disappeared (Ward *et al.*, 2001). In addition to occupational exposure and smoking, VC is present in groundwater from many Superfund sites and some public drinking water in parts per billion amounts as a result of dechlorination of the common groundwater contaminants, trichloroethylene, and perchloroethylene (ATSDR, 2006).

VC DNA Adducts and Mutagenicity

VC is a procarcinogen that requires metabolic activation, with the primary enzyme involved being Cytochrome P450 2E1, which converts VC to chloroethylene oxide (CEO). CEO covalently binds to DNA, inducing four DNA adducts (Fig. 4). The most prevalent DNA adduct is 7-(2-oxoethyl)guanine (OEG), which represents $\sim 98\%$ of the adducts formed by VC. OEG is not promutagenic, i.e., it does not cause mispairing during DNA replication (Barbin *et al.*, 1985). It is lost primarily by chemical depurination, but some repair by glycosylases is also possible. In either case, abasic sites represent a repair intermediate. With daily exposure, OEG adducts reach steady-state concentrations in ~ 7 days and have a $T_{1/2}$ of 4 days (Mutlu, unpublished data). VC also induces three

exocyclic etheno DNA adducts 1, N^6 -ethenodeoxyadenosine (ϵdA), 3, N^4 -ethenodeoxycytosine (ϵdC), and $N^2,3$ -ethenoguanine (ϵG) (Bartsch *et al.*, 1994; Guengerich and Persmark, 1994). ϵG has been measured using gas chromatography/mass spectrometry (GC/MS) and gas chromatography/high resolution mass spectrometry (GC/HRMS) following derivatization with or without immunoaffinity enrichment (Fedtke *et al.*, 1989, 1990a,b; Morinello *et al.*, 2001, 2002a,b). Most recently, ϵG was analyzed following neutral thermal hydrolysis and HPLC enrichment with LC-MS/MS pseudoSRM (Mutlu *et al.*, 2010). Most research analyzing ϵdA and ϵdC has utilized immunoaffinity- ^{32}P -postlabeling (Nair *et al.*, 1995; Reddy, 1993). Recently, an LC-MS/MS method was applied using either immunoaffinity (Ham *et al.*, 2004) or HPLC (Gao, unpublished data) enrichment.

All three etheno adducts are promutagenic, as summarized in Table 4. Mutations have been induced in site-specific mutagenesis assays (Basu *et al.*, 1993; Cheng *et al.*, 1991; Shibutani *et al.*, 1996; Singer *et al.*, 1991; Zhang *et al.*, 1995) as well as following *in vitro* exposure to CEO or chloroacetaldehyde (Guengerich, 1992; Oesch and Doerjler, 1982). The human *K-ras* gene is activated by a GC \rightarrow AT base substitution at the second base of codon 13. This mutation was present in 5/6 human VC-induced angiosarcomas (Marion *et al.*, 1996) and is consistent with the known promutagenic properties of ϵG and ϵdC . Hollstein *et al.* (1994) reported AT \rightarrow TA mutations in the *p53* tumor suppressor gene in 2/4 human hepatic angiosarcomas analyzed in VC workers. These mutations are consistent with the miscoding properties of ϵdA .

Endogenous VC DNA Adducts

Etheno adducts have been identified in unexposed humans and rats in several studies (Barbin *et al.*, 2003; Bartsch *et al.*, 1994; Morinello *et al.*, 2002a; Nair *et al.*, 1995). *In vitro* studies demonstrated that incubating nucleosides or

TABLE 4
Miscoding Properties of VC DNA Adducts

7-(2-Oxoethyl)guanine (OEG)	None
N ² ,3-Ethenoguanine (εG)	G → A
3,N ⁴ -Ethenodeoxycytidine (εC)	C → T
	C → A
	C → G
1,N ⁶ -Ethenodeoxyadenosine (εdA)	A → T
	A → C
	A → G

nucleobases with lipid peroxidation products resulted in the formation of εA, εC, and εG (el Ghissassi *et al.*, 1995; Ham *et al.*, 2000, 2004; Nair *et al.*, 1996). Most recently, endogenous OEG was also identified in [¹³C₂]-VC-exposed rats (Mutlu, unpublished data).

A review of human studies on DNA adducts associated with inflammation and lipid peroxidation was published by Nair *et al.* (2007). This paper includes data from many studies on εdA and εdC, including nondiseased tissues. It reported values ranging from not detected to 29 εdA and εdC adducts per 10⁹ parent bases. Median values of endogenous etheno adducts present in a variety of tissues from 12 human autopsy cases with no known exposure to VC ranged from 1.0 to 2.9 εdA/10⁸ dA and 1.9 to 4.8 εdC/10⁸ dC (Barbin *et al.*, 2003). The study by Barbin also examined aldehydic lesions such as abasic sites and found 3.7–11.3 aldehydic DNA lesions (ADL)/10⁶ nt. Ethenoguanine has been less well studied, with ~15 εG/10⁸ G in both human liver and colon (Hussain *et al.*, 2000; Swenberg *et al.*, 1999).

Several studies have examined DNA from unexposed rodent tissues and demonstrated the presence of endogenous etheno adducts. Using immunoaffinity/³²P-postlabeling, control rat liver had ~0.4–5.8 εdA/10⁹ dA and 0.6–40 εdC/10⁹ dC (Guichard *et al.*, 1996; Nair *et al.*, 2005). Guichard *et al.* (1996) also reported ~2 εdA/10⁸ dA and 9 εdC/10⁸ dC in lung and ~3 εdA/10⁸ dA and 10 εdC/10⁸ dC in kidney. Ham *et al.* (2004) developed an LC-MS/MS assay for εdA and reported ~0.8 εdA/10⁸ dA in control and *Aag* null mouse

liver but 0.1 εdC/10⁸ dC in control mouse lung compared with 0.7 εdC/10⁸ dC in the lung tissues of *Aag* knockout mice. Morinello *et al.* (2002b) utilized immunoaffinity GC-HRMS to first show endogenous εG in rat liver and brain DNA. Liver had ~4 εG/10⁸ G, whereas brain had ~5.3 εG/10⁸ G in control animals. It is of interest that human tissues tended to have greater amounts of endogenous etheno adducts than rodents.

Relationships of Exogenous/Endogenous VC DNA Adducts

Several studies have compared endogenous DNA adducts identical to those induced by VC (Gao, unpublished data; Mutlu *et al.*, 2010). The most informative studies have utilized 1100 ppm [¹³C₂]-VC exposures for 5 or 20 days (6 h/day) that provided accurate information on both endogenous and exogenous DNA adducts in the same DNA sample (Gao, unpublished data; Morinello *et al.*, 2002a,b; Mutlu *et al.*, 2010). Morinello *et al.* (2002b) examined adult rat liver and brain DNA and demonstrated that 5.2 ± 1.2 endogenous εG/10⁸ G were present in hepatocyte DNA, whereas 55 ± 4.0 [¹³C₂]-εG/10⁸ G were formed following 5 days of exposure to 1100 ppm [¹³C₂]-VC (6 h/day) and 110 ± 20 [¹³C₂]-εG/10⁸ G were formed following 20 days of exposure. In contrast, 5.4 ± 1.2 endogenous εG/10⁸ G were present in brain, but no [¹³C₂]-εG was detectable following 5 or 20 days of exposure. This strongly supports the more comprehensive epidemiology studies that did not find a causal association between brain tumors and VC exposure. We hypothesize that the chemical instability of CEO formed in the liver does not allow it to be transported to the brain where it can form DNA adducts, whereas the endogenous adducts are formed from oxidative stress arising within the brain.

Table 5 provides quantitative data for OEG, εG, and εdA from a study that also exposed rats to 1100 ppm [¹³C₂]-VC for 5 days (6 h/day) (Gao, unpublished data; Mutlu, unpublished data; Mutlu *et al.*, 2010). Results similar to those of Morinello *et al.* (2002b) were shown in adult liver DNA (4.1 ± 2.8 endogenous εG/10⁸ G vs. 18.9 ± 4.9 [¹³C₂]-εG/10⁸ G). Using sequential sacrifices 2, 4, and 8 weeks later, they demonstrated that [¹³C₂]-εG adducts had little or no repair, with a half-life of 150 days. In contrast, endogenous hepatic OEG was present at

TABLE 5
Relative Amounts of Endogenous and Exogenous DNA Adducts in Liver DNA from Rats Exposed to [¹³C₂]-VC (1100 ppm, 6 h/Day, 5 Days)

	[¹² C ₂]-OEG/10 ⁵ Gua	[¹³ C ₂]-OEG/10 ⁵ Gua	[¹² C ₂]-N ² ,3-εG/10 ⁸ Gua	[¹³ C ₂]-N ² ,3-εG/10 ⁸ Gua	[¹² C ₂]-1, N ⁶ -εdA/10 ⁸ dA	[¹³ C ₂]-1, N ⁶ - εdA/10 ⁸ dA
Adult rats at end of exposure	0.2 ± 0.1	10.4 ± 2.3	4.1 ± 2.8	18.9 ± 4.9	4.9 ± 0.6	5.1 ± 0.6
2 Weeks postexposure	0.1 ± 0.03	0.4 ± 0.3	3.7 ± 3.1	14.2 ± 4.2	8.6 ± 0.9	n.d.
4 Weeks postexposure	0.2 ± 0.04	0.1 ± 0.06	3.1 ± 1.0	16.9 ± 1.6	6.2 ± 1.3	n.d.
8 Weeks postexposure	0.2 ± 0.07	n.d.	3.7 ± 1.5	13.2 ± 2.5	4.1 ± 0.5	n.d.

Note. n.d., not detected.

2 ± 1 OEG/ 10^6 G at the end of the 5-day exposure and over the next 8 weeks. [$^{13}\text{C}_2$]-OEG was present at the end of the 5-day exposure at 104 ± 23 [$^{13}\text{C}_2$]-OEG/ 10^6 G, 4 ± 3 [$^{13}\text{C}_2$]-OEG/ 10^6 G 2 weeks later and was not detectable 4 or 8 weeks postexposure. The half-life of [$^{13}\text{C}_2$]-OEG was calculated to be 4 days. In our laboratory, Gao developed a nano-UPLC-MS/MS assay for ϵ DA to analyze same tissues (Gao, unpublished data). She found 4.9 ± 0.6 endogenous ϵ DA/ 10^8 dA in the 1100 ppm [$^{13}\text{C}_2$]-VC-exposed rat liver compared with 5.1 ± 0.6 [$^{13}\text{C}_2$]- ϵ DA/ 10^8 dA. No [$^{13}\text{C}_2$]- ϵ DA adducts could be detected in liver samples from rats killed 2, 4, or 8 weeks postexposure. The study design did not allow accurate calculation of the half-life of [$^{13}\text{C}_2$]- ϵ DA adducts, as the postexposure times were too long. This is consistent with other data suggesting that ϵ DA is rapidly repaired with a half-life of ~ 24 h (Ham *et al.*, 2004). These [$^{13}\text{C}_2$]-VC studies are the only data that discriminate between endogenous and exogenous VC adducts, as all other studies have been measuring a mixture of endogenous and exogenous adducts. It is clear that markedly different half-lives exist for ϵ DA, OEG, and ϵ G.

Application of Data on Exogenous/Endogenous Adducts to VC Risk

The hepatic angiosarcomas observed in workers exposed to high workplace concentrations of VC provide compelling evidence of its carcinogenicity in humans. A major question remains nonetheless regarding the nature of the exposure-response relationship: is it linear or not? Are these tumors a high, sustained dose-only phenomenon, as has been observed in the workplace, or might they also manifest at lower, intermittent doses? Interestingly, the three promutagenic etheno adducts of CEO that arise from VC metabolism exhibit markedly different endogenous/exogenous ratios that can be traced back to their markedly different half-lives, with the etheno-G adduct having by far the longest half-life (in rats) of 150 days. Thus, continuous exposure for extended periods can lead to higher exogenous/endogenous ratios for ϵ G that would translate into greater predicted relative risks using the bottom-up approach. In contrast, OEG will reach steady-state levels in 7–10 days, and ϵ DA and ϵ dC will not increase over exposure time. More research is needed to sort out the implications for human cancer risk of short-lived, rapidly repaired adducts in relation to long-lived, poorly repaired adducts.

ETHYLENE OXIDE

Ethylene oxide (EO) has been classified as a known human carcinogen by IARC. This classification was based on limited evidence in humans for LEU, breast, and stomach cancer, sufficient evidence for carcinogenicity in experimental animals, and an array of genetic toxicology data in humans and animals (IARC, 1994). Ethylene oxide is a genotoxic chemical that

causes both chromosomal and gene mutations. To date, the types of DNA damage responsible for creating EO mutations remain unknown.

Ethylene Oxide DNA Adducts and Mutagenicity

Ethylene oxide is a direct-acting alkylating agent that binds to DNA at several positions (Fig. 5). Numerous groups have conducted research on the DNA and globin adducts of EO, with most data collected during the last 20 years. The advancement of analytical methods such as derivatization and MS, HPLC with fluorescence detection, and the application of ^{32}P -postlabeling of DNA adducts provided methods with adequate sensitivity to accurately measure the DNA and globin adducts of EO. Törnqvist *et al.* (1986) developed the Edman degradation method for GC/MS analysis of N-terminal valine adducts of EO in 1986. This method clearly demonstrated the presence of hydroxyethylvaline adducts in unexposed humans and animals. Subsequently, GC/MS and GC/HRMS methods for detecting N7-(2-hydroxyethyl)guanine (7HEG), the major DNA adduct formed by EO, were developed (Föst *et al.*, 1989; Wu *et al.*, 1999), and ^{32}P -postlabeling of DNA was used to determine EO DNA adducts (Eide *et al.*, 1999). More recently, LC-MS/MS and HPLC coupled with accelerator MS have been used to measure exogenous 7HEG (Marsden *et al.*, 2007; Rusyn *et al.*, 2005). Whereas 7HEG makes up $\sim 95\%$ of the DNA adducts, EO forms several additional minor DNA adducts including N3-(2-hydroxyethyl)dA, N3-(2-hydroxyethyl)dU, and O⁶-(2-hydroxyethyl)dG (La *et al.*, 2010). 7HEG is not considered a promutagenic DNA adduct but can depurinate and produce an abasic site that could be mutagenic if present during DNA replication. However, inhalation exposures of rats to 100 ppm EO for 4 weeks (6 h/day, 5 days/week) did not result in an increase in abasic sites (Rusyn *et al.*, 2005). When mice were exposed to ethylene or EO by inhalation for 4 weeks (6 h/day, 5 days/week) and HPRT mutant frequencies were determined, there was no increase in mutations at 40, 1000, or 3000 ppm ethylene, which is equivalent to 4.5, 9, or 10 ppm EO. The lowest exposure that increased HPRT mutant frequencies was 50 ppm EO (Swenberg *et al.*, 2008). More recently, EO mutagenicity was evaluated in the pSP189 shuttle vector being replicated in human Ad293 cells (Tompkins *et al.*, 2009). Even when plasmids containing up to 29,000 7HEG adducts/ 10^8 nt were evaluated, no other EO DNA adducts could be detected using sensitive LC-MS/MS methods and no increases in mutations were found. Only when very high exposures to EO were used and N1-hydroxyethyl dA (N1-HEdA), O⁶-hydroxyethyl dG (O⁶-HEdG), and N3-hydroxyethyl dU (N3-HEdU) could be measured were significant increases in mutations apparent. The half-lives of these three adducts are much shorter than 7HEG because of active DNA repair pathways. This plethora of data suggests that EO is a relatively weak mutagen.

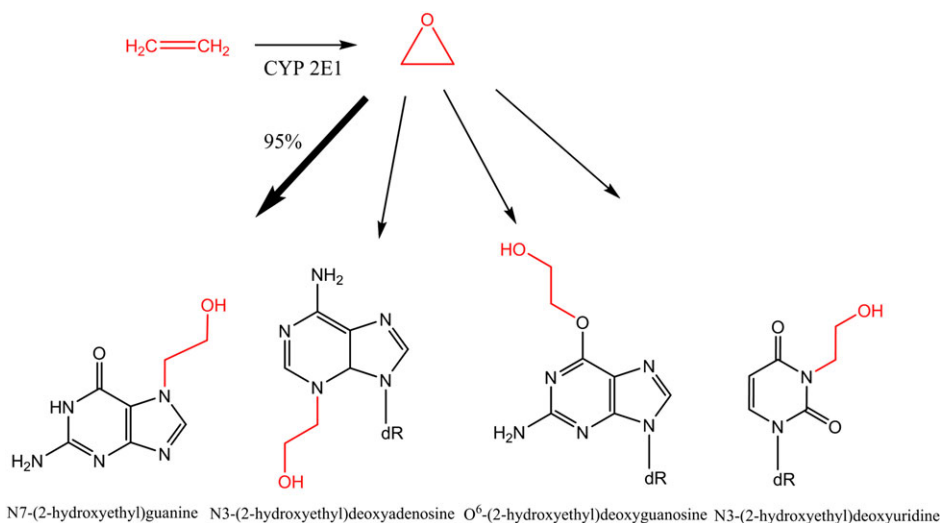


FIG. 5. Metabolism of ethylene and formation of DNA adducts from EO.

Endogenous Ethylene Oxide DNA Adducts

Endogenous amounts of 7HEG in tissues from unexposed rats and mice range from 1 to 4 7HEG/ 10^8 nt (Marsden *et al.*, 2007; Wu *et al.*, 1999), whereas human liver had 58.5 ± 21.4 7HEG/ 10^8 nt and human lymphocytes had 48 ± 31 7HEG/ 10^8 nt (Wu *et al.*, 1999). Thus, humans appear to have nearly 10-fold higher amounts of endogenous 7HEG than are found in rats and mice. Endogenous 7HEG arises from multiple sources, including the generation of ethylene from methionine oxidation, lipid peroxidation, and bacterial metabolism. Endogenous ethylene is metabolized to EO in the liver, circulates in the blood to expose all tissues, and is exhaled in expired breath. Of considerable interest is the recent study by Marsden (Marsden *et al.*, 2009), who found that ip injections of [$^{14}\text{C}_2$]-EO not only formed [$^{14}\text{C}_2$]-labeled exogenous 7HEG but also caused dose-related increases in unlabeled, endogenous 7HEG. They went on to demonstrate that the increase in endogenous 7HEG was because of increased oxidative stress. This may be the result of competition for detoxication pathways between endogenous and injected [$^{14}\text{C}_2$]-EO. Additional evidence for endogenous 7HEG was provided by the demonstration of 7HEG in human urine (Cushnir *et al.*, 1993; Huang *et al.*, 2008). The amounts of 7HEG in urine were lowest in nonsmokers with no occupational exposure to EO.

Relationships of Exogenous/Endogenous Ethylene Oxide DNA Adducts

Numerous studies have measured 7HEG in various tissues of rats, mice, and humans exposed to EO. What must be clearly understood is that such biomarker measurements actually represent exposure to both endogenous and exogenous EO. To

some degree, the source can be ferreted out by comparing exposed individuals to unexposed controls. However, the use of stable isotope exposures, followed by adduct purification and MS, is the only way to measure both endogenous and exogenous 7HEG in DNA at the same time. To date, this has not been reported for EO. As mentioned earlier, Marsden *et al.* (2007, 2009) exposed mice to [$^{14}\text{C}_2$]-EO ip at doses of 0, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, or 0.1 mg/kg per day for 3 consecutive days and harvested the tissues 4 h after the last dose. The authors concluded that the responses in liver, spleen, and stomach were dose dependent and not significantly nonlinear. The authors also pointed out that several of the lowest doses induced numbers of adducts that were below the limit of quantitation for the AMS method ($\sim 20\text{--}50$ 7HEG/ 10^{12} nt). To put that in perspective with endogenous 7HEG, this amount is 1000 times lower than endogenous 7HEG observed in control rodent tissues and 10,000 times lower than measured in normal human tissues. The molecular dose of 7HEG created by such low exposures implies that only 1/1000 7HEG adducts in a mouse and only 1/10,000 adducts in a human would arise from exogenous exposure, assuming that rodents and humans form equivalent molecular doses from equal exposures. The endogenous EO adducts outnumber the exogenous adducts by such a vast margin that the exogenous adducts are not likely to be causal for EO-induced mutations or cancer. When looked at from the perspective of the total number of endogenous DNA adducts in a cell, it is clearly implausible.

Application of Data on Exogenous/Endogenous Adducts to EO Risk

It is worth exploring implications of the bottom-up approach that was taken herein for formaldehyde risk assessment in the case of EO. Although the adduct data are more limited,

potentially useful inferences can be drawn from what information is presently available. For example, it was noted previously that the LOD for exogenous 7HEG adducts using AMS (~20–50 adducts per 10^{12} nt) is approximately 1000-fold lower than the measured endogenous amounts of these adducts in human tissues. This implies, even for a simple linear model of cancer risk, that the incremental risk above background arising from exposures that produce fewer adducts than can be currently detected must likewise be less than 1 in 1000. Estimated relative risks (risk at a given exposure level divided by the background risk) would thus be conservatively constrained to be less than 0.001, a value far smaller than could ever be detected in retrospective cohort mortality studies of exposed workers. This “prediction” suggests that the limited evidence in humans of increased LEU, breast, and stomach cancer may in fact be false-positive findings. Of course, many caveats apply to such extrapolations, and additional dose-response data will be needed to support or refute the numerous assumptions that are required to produce them.

CONCLUSIONS

Over the last 15 years, many researchers have investigated relationships between DNA damage and repair, mutagenesis, and carcinogenesis. This was also the era of discovery of an ever-growing literature on endogenous DNA damage. During this same period, risk assessment practices have gone from minimal incorporation of mechanistic data and reliance on the linearized multistage model for low-dose risk assessment to strong emphasis on Mode of Action and the incorporation of such scientific data into the risk assessment process. However, this approach is not uniformly accepted, as some scientists and risk assessors support the use of linear risk assessment with little regard for the incorporation of science into the decision making process, an approach outlined in *Science and Decisions* (NRC, 2009). This paper highlights data for three chemicals listed by IARC as known human carcinogens, which also form DNA adducts identical to endogenous DNA damage. These examples provide great insight into issues related to evaluations of cancer causality and low-dose risk assessment.

The carcinogenicity of inhaled formaldehyde was discovered 30 years ago (Swenberg *et al.*, 1980). Since that time, one of the richest understandings of the key events involved in carcinogenesis has come forth. Despite this, the recent draft risk assessment of the U.S. EPA has ignored much of the science and has proposed risks in tissues with no mechanistic support that are magnitudes above what is plausible. The recent paper of Lu *et al.*, (2010a) was highlighted by this journal and the editor wrote a special commentary (Lehman-McKeeman, 2010) that stated: “In their present work, Lu *et al.* have provided the first definitive evaluation of DNA adducts arising from endogenous and exogenous formaldehyde, a novel and important contribution to the scientific literature. Moreover,

they have generated the type of data that should be given strong consideration by toxicologists and risk assessors as the data and their interpretations are used to improve the scientific basis of human health risk assessment.” We have taken the challenge to begin applying the type of data generated in Lu *et al.*, (2010a) and conducted additional research to better address complementary aspects of molecular dosimetry, species differences, and DNA adduct persistence and the fact that formaldehyde is an essential biochemical of every living cell. This information was then utilized to generate a public health conservative approach to risk assessment that made use of the new and previously known biology and toxicology related to formaldehyde and cancer.

VC and EO also have clearly been shown to induce DNA adducts identical to endogenous DNA adducts in experimental animals and humans. Although less studied than formaldehyde, the available information demonstrates that similar approaches could be applied to these chemicals.

Going beyond chemicals that form identical endogenous and exogenous DNA adducts, there are approximately 50,000 endogenous DNA lesions in every living cell. This steady-state amount of DNA damage has long been considered to represent a major source of cancer and other diseases. Despite this, it has not been taken into consideration in risk assessments in determining what exposures actually produce disease. For that matter, epidemiology has not adequately utilized information on biomarkers to assist in the determination of causality. Such data can help support or refute causal associations.

We propose that this approach is set in an embryonic state of knowledge but strongly believe that public health and effective regulation of chemicals will be strengthened by science-based determinations of causality and promulgations of risk. To achieve this, we must have better data on low-dose mutagenicity, improved Mode of Action understanding that covers high- to low-dose ranges, and species differences and similarities. We can then move forward, embracing public health protective actions that are based on science. This includes the application of reasonable additional factors for susceptible individuals.

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