

**U.S. Environmental Protection Agency Science Advisory
Board Chemical Assessment Advisory Committee (CAAC)
Ethylene Oxide Review Public Meeting**

(Comments related to Charge Question #5)

**Richard J. Albertini M.D., Ph.D.
Professor Emeritus, Medicine
University of Vermont**

Speaking as a consultant to the American Chemistry Council

November 18-20, 2014

ENDOGENOUS VERSUS EXOGENOUS DNA ADDUCTS

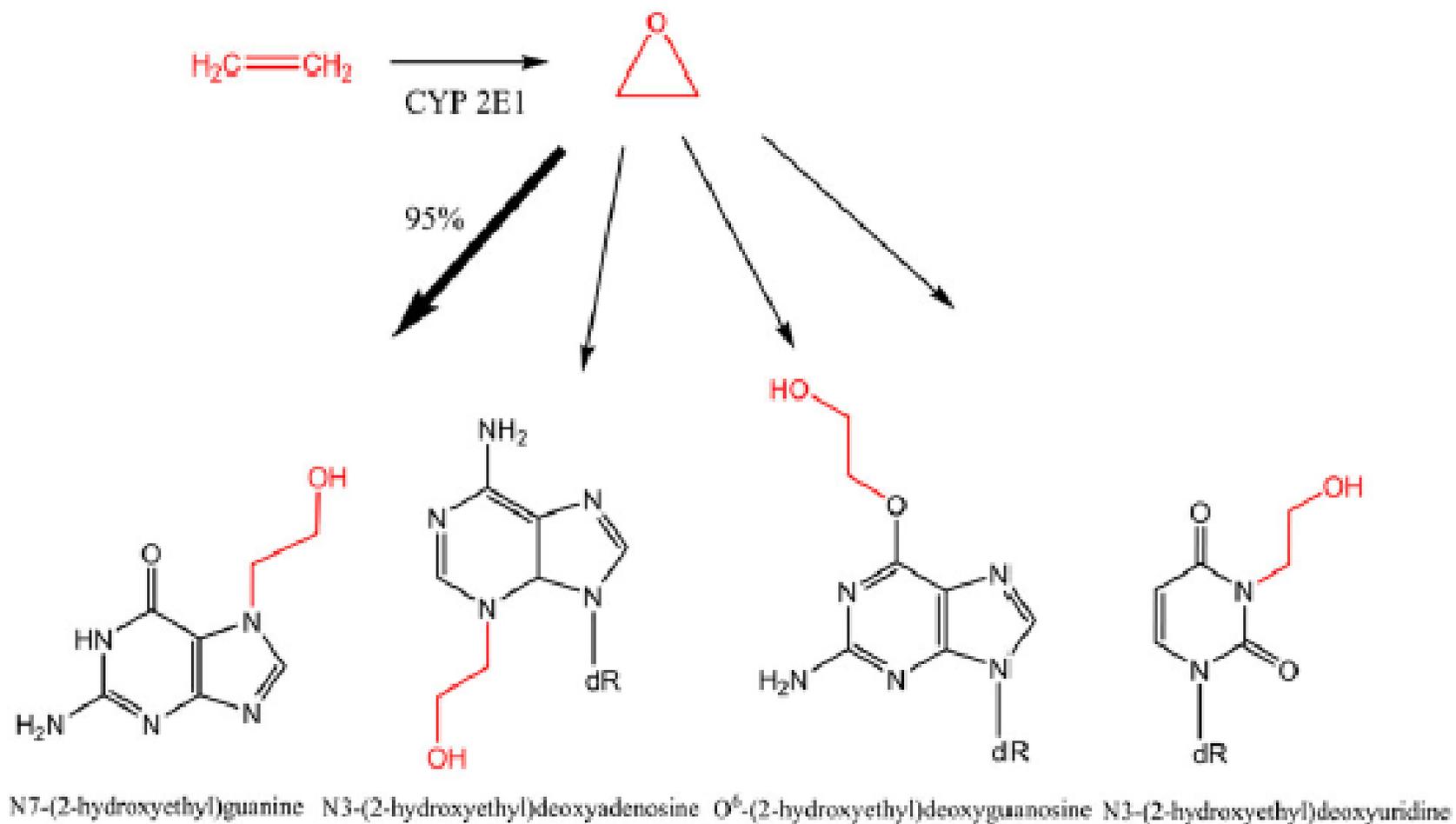


FIG. 5. Metabolism of ethylene and formation of DNA adducts from EO.

ETHYLENE OXIDE

DNA Adducts

N7G (81%)*

N3A (10%)

N1A (7%)

O⁶G (0.4%)

N⁶A (small amt)

N3C→N3U (small amt)

N3T (small amt)

** N7G = marker adduct*

EO: Alternate Mutagenic Mechanisms: (Indirect Mutagenesis)

Oxidative Stress

- *Marsden et al. (2009)*

High dose effect; mimicked by H₂O₂; produces N7HEdG adducts

Mitogenesis

- *Recio et al. (2004)*

lacI mutations in testes of mice after 48 wk inhalation exposure with background mutation spectrum indicating amplification of pre-existing mutants.

- *Parsons et al. (2013)*

Ras mutations in lungs of mice after 4 wk inhalation exposures with background mutation spectrum suggesting amplification of pre-existing mutants.

EO: Pro-mutagenic adduct(s) in target tissue?

N7-hydroxyethyl-dG is **NOT A PROMUTAGENIC ADDUCT***

- *Tompkins et al. (2009) Mutation Research*
(pSP189 shuttle vector replicated in human Ad 239 cells)
- *Philippins et al. (2014) DNA repair*
(N7dG adduct in plasmids transformed into bacteria)

* Nor do abasic sites accumulate (*Rusyn et al., 2005*)

EO dose, DNA adducts and Mutation induction (*Tompkins et al. 2009*)

132

E.M. Tompkins et al. / Mutation Research 678 (2009) 129–137

Table 1

Summary of mean plasmid DNA adduct levels and relative mutation frequencies for Studies 1 and 2.

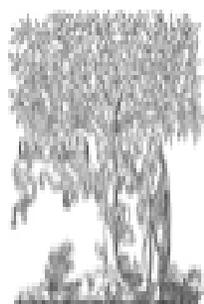
EO dose	DNA adducts/10 ⁶ nucleotides			Relative mutation frequency ^a	
	N7-HEG ^b		N1-HEdA	O ⁶ -HEdG	
Control 1	4.6	4.0	nd ^c	nd	1.0
Control 2	8.9	2.5	nd	nd	3.0
10 μM	12.2	8.4	nd	nd	0.8
20 μM	12.8	11.3	nd	nd	2.6
50 μM	8.0	6.4	nd	nd	1.7
100 μM	33.2	9.9	nd	nd	1.5
500 μM		32.3 ^d	nd	nd	1.9
1000 μM	118.2	41.7	nd	nd	0.7
2000 μM	290.0	50.7	nd	nd	2.0
10 mM		2880	150	2.8	3.14
30 mM		3520	680	7.5	5.34
50 mM		5030	720	13.4	NA
100 mM		9800	2240	16.4	NA

^a The mean relative mutation frequency (per 10⁴ colonies) is normalised to the respective Solvent Control 1 in each set of reactions which is set at a value of 1.0.

^b N7-HEG adduct levels in Study 1 are from two sets of experiments, giving two values for each EO dose where the first figure listed is from Set 1 and the second from Set 2.

^c nd signifies that the particular adduct was not detectable at this dose.

^d Adducts were only quantified in plasmid from the 2nd dosing experiment for 500 μM EO. NA indicates that mutation frequency data were not acquired at these doses due to the high degree of plasmid fragmentation.

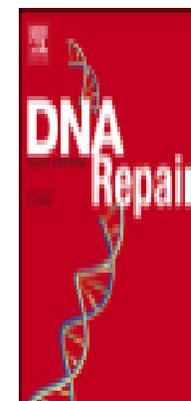


ELSEVIER

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair



Brief Communication

Ethylene oxide and propylene oxide derived N7-alkylguanine adducts are bypassed accurately *in vivo*



Gaëlle Philippin^a, Jean Cadet^b, Didier Gasparutto^b, Gerard Mazon^{a,1}, Robert P. Fuchs^{a,*}

^aCancer Research Center of Marseille, CNRS, UMR7258 Genome Instability and Carcinogenesis (équipe labellisée Ligue Contre le Cancer) Inserm, U1068, Pauli-Calmettes Institute, Aix-Marseille Univ, F-13009 Marseille, France

^bLésions des Acides Nucléiques, INAC/SCIB – UMR-E3 CEA/UJF, CEA Grenoble 17 avenue des Martyrs, F-38054 Grenoble Cedex 9, France

N7HEdG Adduct is not mutagenic

Table 2

Single colony sequencing results: for each adduct, namely *N7-mG*, *N7-heG* and *N7-hpG*, as well as for control *G*; individual colonies from two independent experiments were sequenced (exp 1 and exp 2). For each experiment the number of wild type and mutant sequences over the total number of colonies sequenced is indicated. The last two columns indicate the total number of mutant over wild type colonies and the estimated mutation frequency, respectively. As no single mutant colony could be detected the mutation frequency appears to be less than 0.3% for all of *N7*-adducts under examination. For each set of sequenced colonies a small number of sequences either failed to give readable results or corresponded to plasmid P1 (data not shown). The presence of plasmid P1 is due to a small amount of parental plasmid that is carried over during the construction.

	# Wild type/total exp 1	# mutant/total exp1	# Wild type/total exp 2	# mutant/total exp2	Mutant/wild type exp (1+2)	Mutation frequency (%)
<i>G</i>	166/166	0/166	192/192	0/192	0/358	<0.28%
<i>N7-mG</i>	172/172	0/172	163/163	0/163	0/335	<0.30%
<i>N7-heG</i>	169/169	0/169	159/159	0/159	0/328	<0.30%
<i>N7-hpG</i>	165/165	0/165	170/170	0/170	0/335	<0.30%

Ethylene Oxide Mutagenicity

- Although positive in many systems, EO is a **weak** mutagen.

Genetic Activity Profile shows that average lowest effective exposure concentrations required to give positive results *in vitro* were between 1.0 and 10.0 µg/ml (23 to 230 µM)*

EO blood levels of this magnitude (~ 23 µM) require inhalation exposures > 150 ppm for four hours in mice**

- Abundant evidence of **non-linearity (thresholds)** at low doses.

Adducts:

Marsden *et al.*, (2009)

Mutations:

Nivard *et al.*, (2003) *Drosophila*

Walker *et al.*, (2000) *Hprt* mammalian *in vivo*

Recio *et al.*, (2004) *LacI* mammalian *in vivo*

Tompkins *et al.*, (2009) Plasmid *in vitro* human cells

LeBlond *et al.*, (2012) MN mammalian *in vivo*

* = Waters *et al.*, 1998

** = Brown *et al.*, 1998

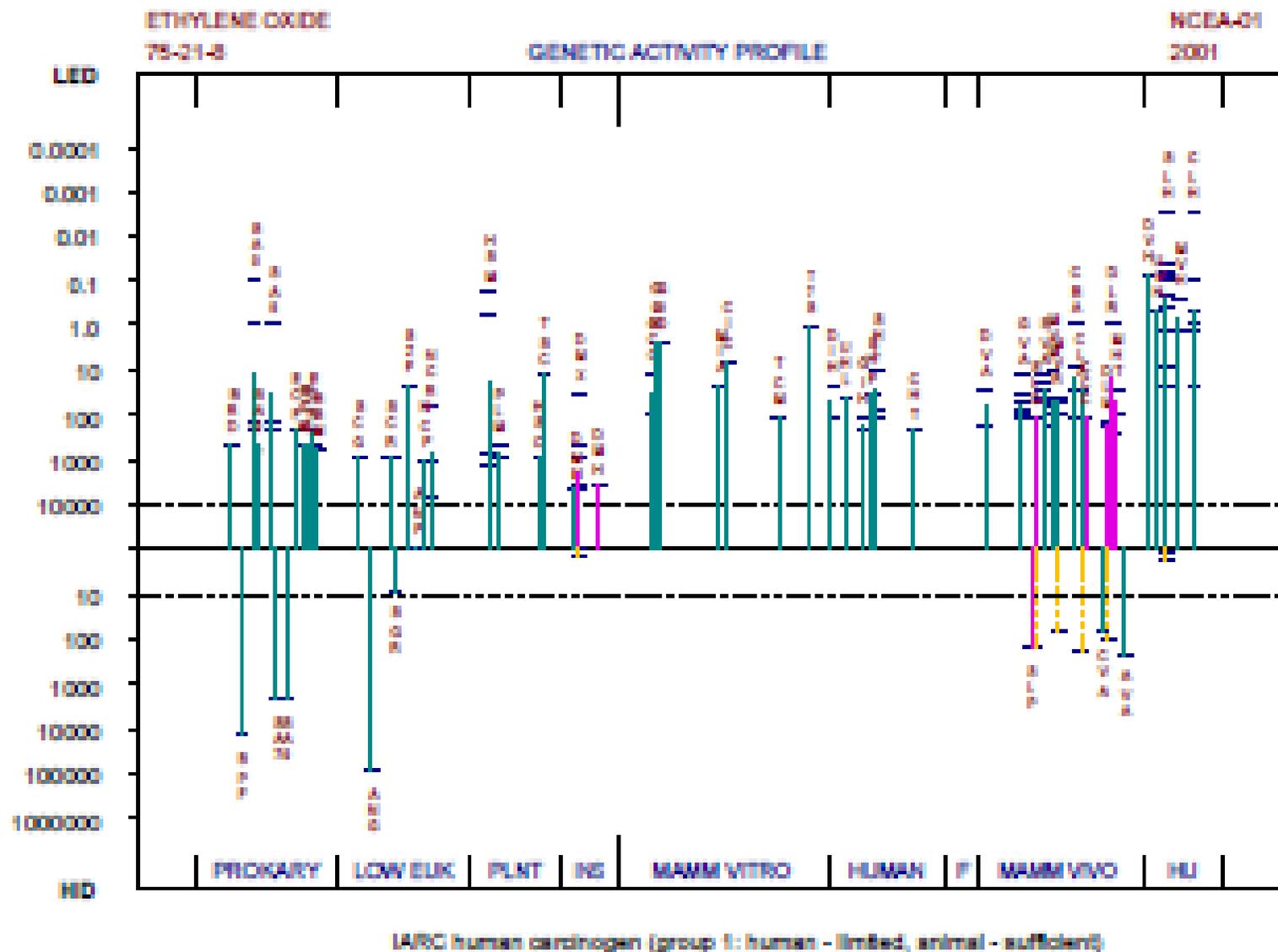
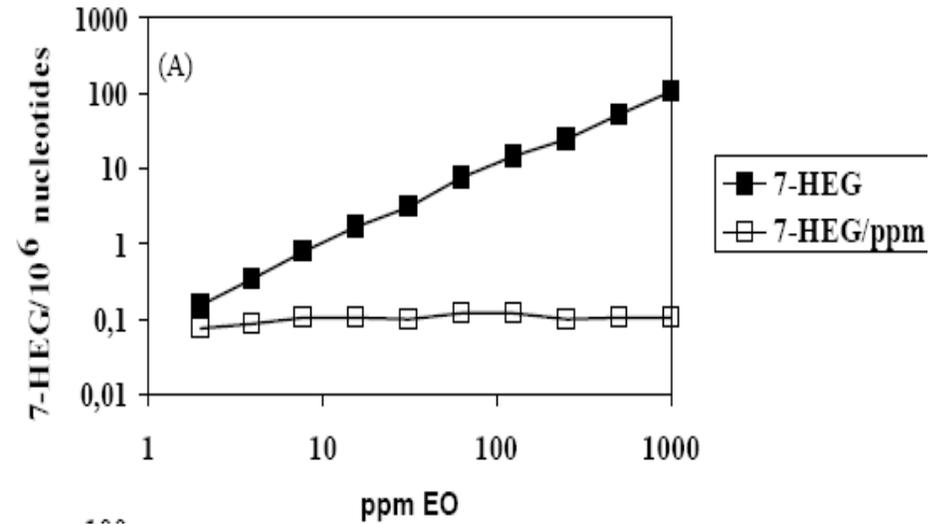
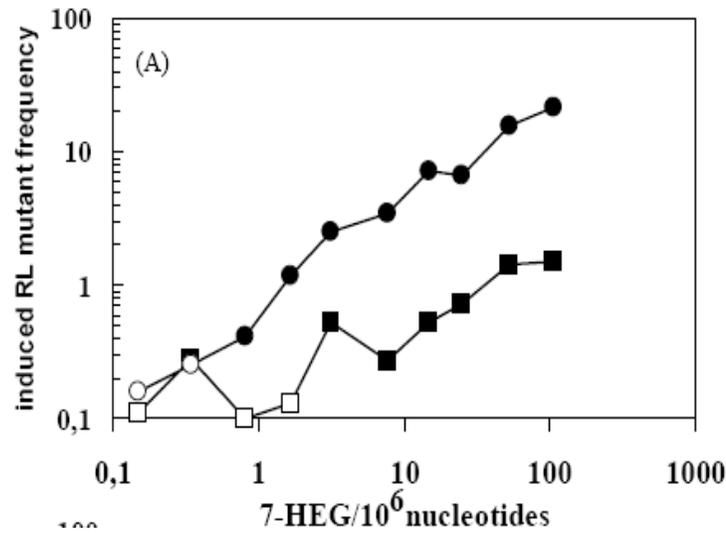


Figure 3-3. Display of 203 data sets, including bacteria, fungi, plants, insects, and mammals (in vitro and in vivo), measuring the full range of genotoxic endpoints. [This is an updated version of the figure in [IARC \(1994b\)](#)]

Mutagenic activity of ethylene oxide and propylene oxide under *XPG* proficient and deficient conditions in relation to *N*-7-(2-hydroxyalkyl)guanine levels in *Drosophila*

Madeleine J.M. Nivard^{a,*}, Kamila Czene^{b,c}, Dan Segerbäck^b, Ekkehart W. Vogel^a



ETHYLENE OXIDE

**WEAK CARCINOGENICITY HIGHLY CORRELATED WITH WEAK
MUTAGENICITY**

(Vogel et al., 1998)

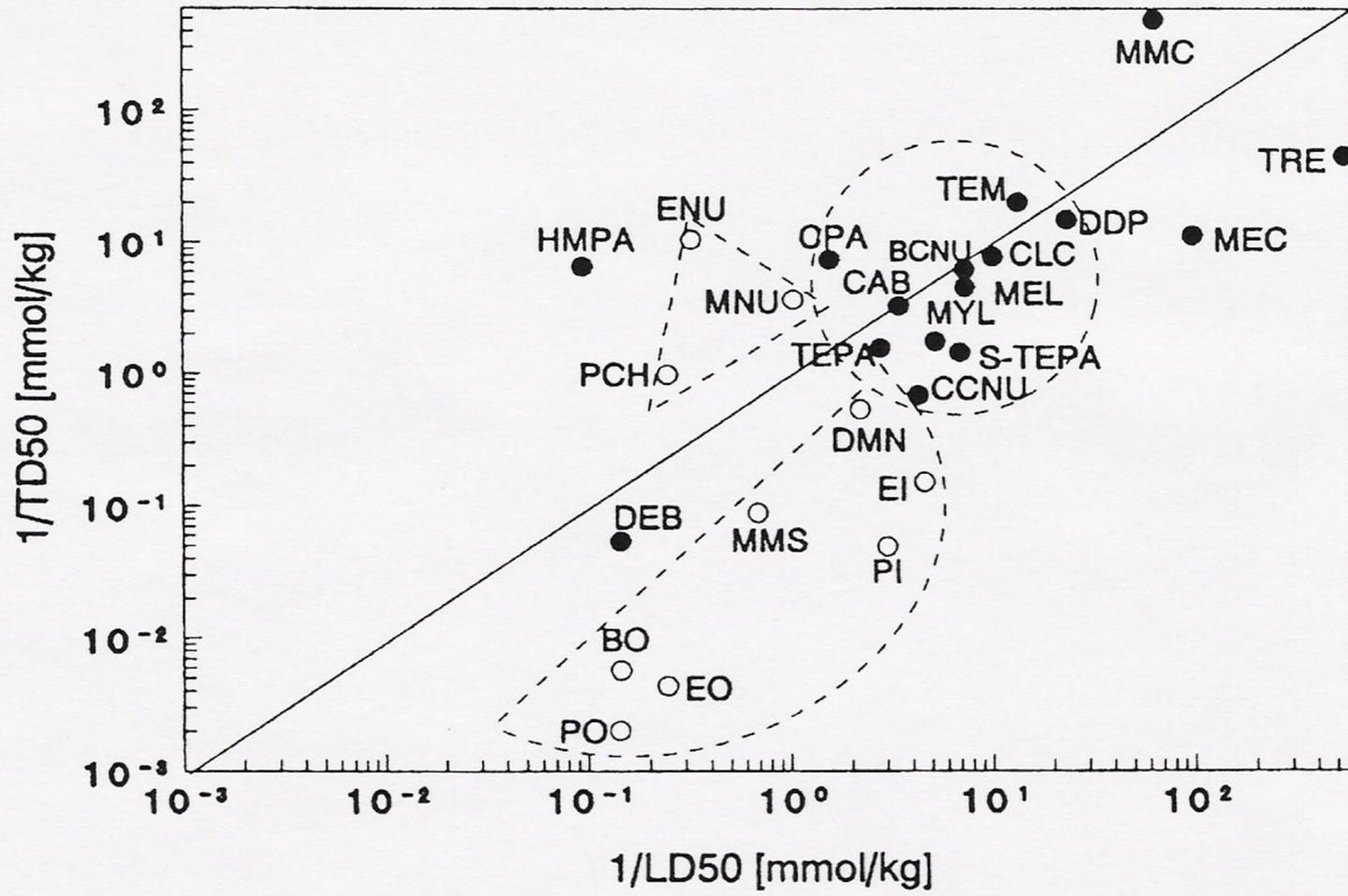
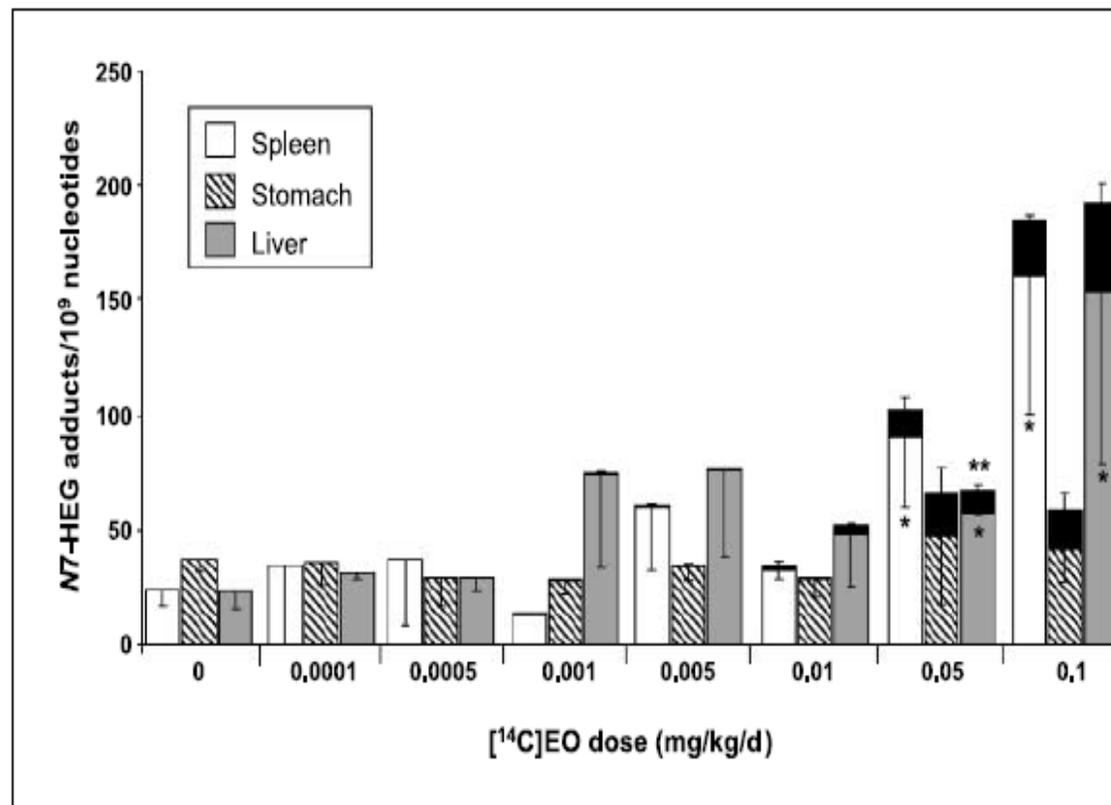
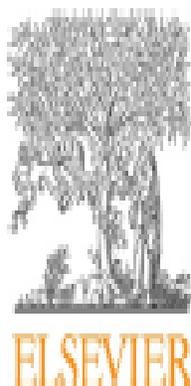


Fig. 12. Carcinogenic potency ($1/TD_{50}$, life-time doses) of alkylating agents in rodents in comparison with acute toxicity ($1/LD_{50}$).

DNA Adducts versus EO Dose: (Marsden et al. 2009)

Figure 2. Contribution of endogenously and exogenously derived N7-HEG to the total adduct level in tissues of [¹⁴C]EO-treated rats. Endogenous adducts were determined by LC-MS/MS. Exogenous ¹⁴C-labeled adducts were quantified by AMS and are shown as black bars on top of bars representing endogenous adduct levels. Columns, mean of three animals per group; bars, SD. *, $P < 0.05$, the level of endogenous N7-HEG in tissues of [¹⁴C]EO-treated rats is significantly higher than the corresponding background level in control animals; **, $P < 0.05$, the total level of adducts (endogenous plus exogenous) is significantly higher than the level of endogenous adducts alone in a particular tissue.



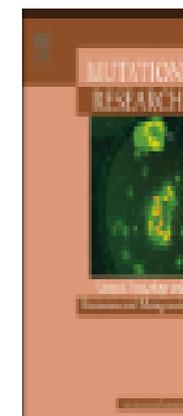


Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres

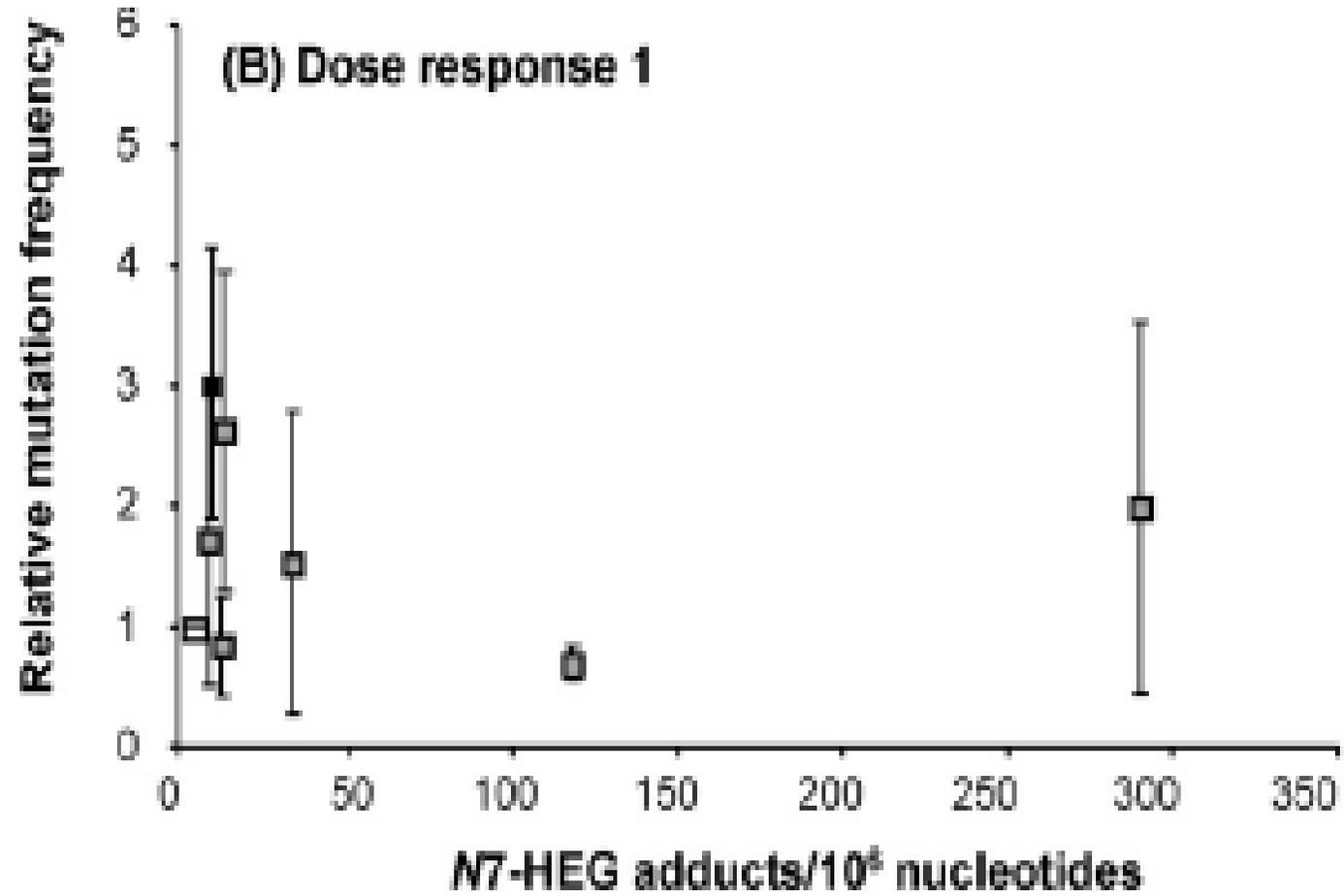


Mutagenicity of DNA adducts derived from ethylene oxide exposure in the pSP189 shuttle vector replicated in human Ad293 cells

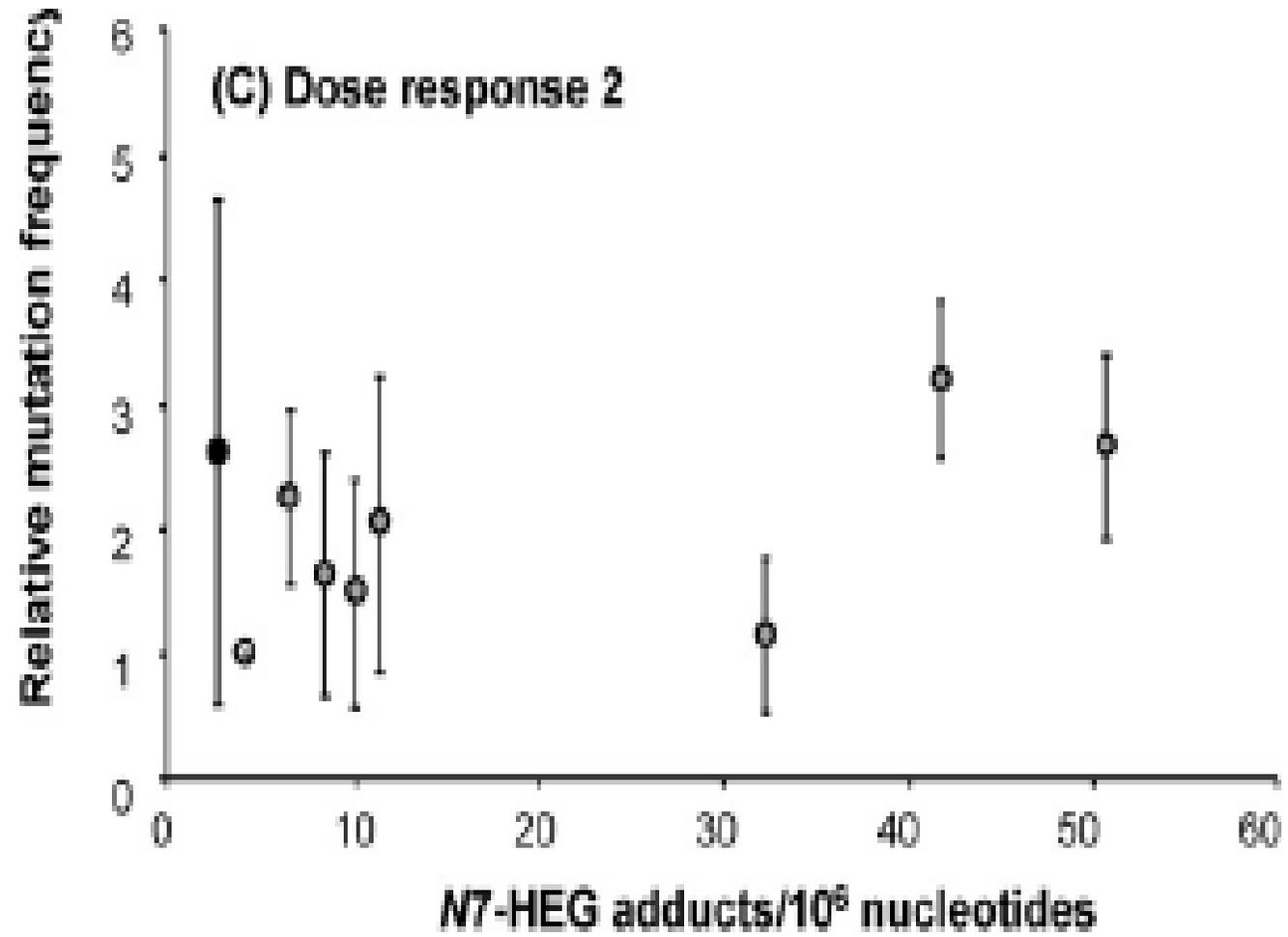
Elaine M. Tompkins, Keith I.E. McLuckie, Donald J.L. Jones, Peter B. Farmer, Karen Brown *

Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, LE2 7LX, UK

Mutation induction versus DNA adducts (*Tompkins et al. 2009*)



Mutation induction versus DNA adducts (*Tompkins et al., 2009*)

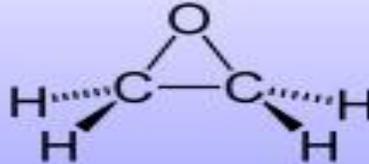


“Under refined reaction conditions using higher EO concentrations capable of inducing detectable levels of *N1-HEdA*, *O6-HEdG* and *N3-HEdU* along with *N7-HEG*, there was a significant dose-related increase in relative mutation frequency above background (3.76- and 5.30-fold at 10 and 30mM, respectively). EO treatment appeared associated with an elevated frequency of GC→CG mutations and the occurrence of substitutions at AT base pairs. Additionally, there was a distinct GC→TA mutational hotspot in the 10mM EO spectrum. Overall, the results suggest a certain level of promutagenic adducts must be attained before mutations become detectable above background, indicating that *N7-HEG* is not a promutagenic lesion, and support a role for the minor products of DNAhydroxyethylation in the generation of base substitutions by EO”.

© 2009 Elsevier B.V. All rights reserved

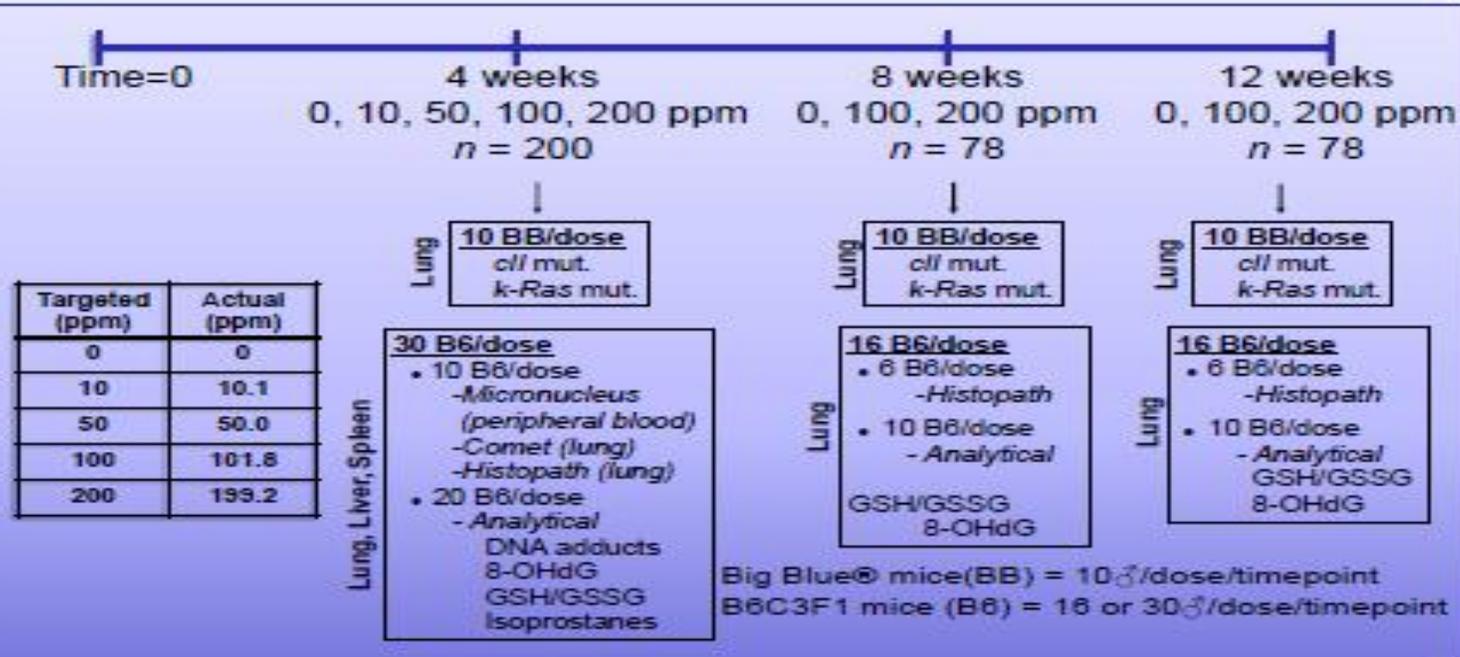
Tompkins *et al.* 2009: excerpt from abstract

Background & Study Design



Ethylene Oxide (EO)

- Colorless, flammable, highly reactive gas used directly or as a reaction product
- *In vitro* and *in vivo* evidence of DNA adducts
- *In vivo* evidence of mutagenicity and clastogenicity
- Alveolar/bronchiolar adenomas and carcinomas in male B6C3F1 mice at 50 and 100 ppm
- This work was sponsored by the Lower Olefins Sector Group and Ethylene Oxide and Derivatives Producers' Association of Cefic, The European Chemical Industry Association



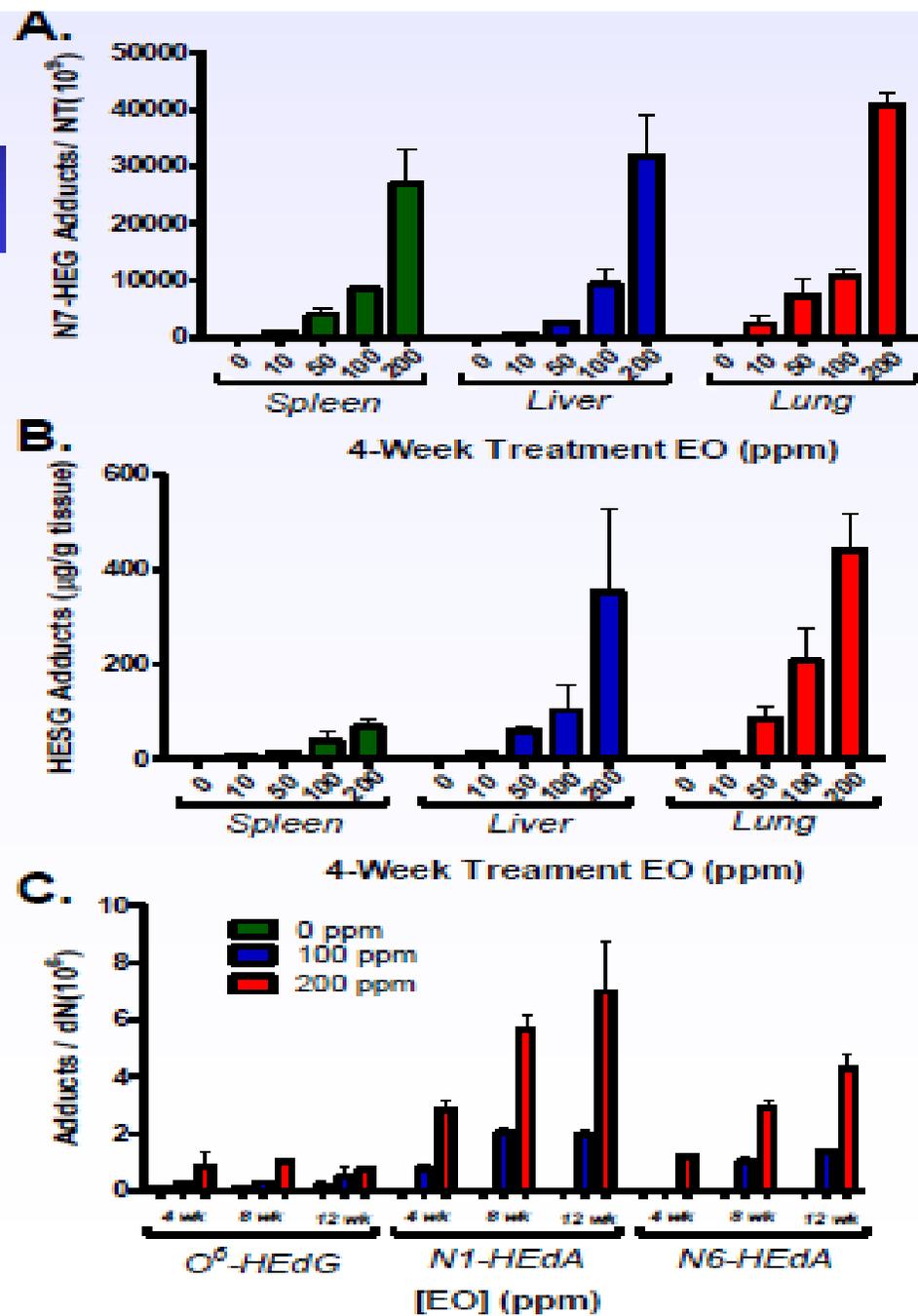


Figure 2. Alkylated DNA and Glutathione Protein Adducts as Biomarkers of Exposure Were Robustly Increased by EO Treatment in All Tissues Analyzed. Levels of DNA adducts associated with alkylated DNA or protein were analyzed by LC/MS and are presented as a function of concentration and duration of exposure. (A) N7-HEG [4 wks exposure; spleen, liver, lung], (B) HESG protein adducts [4 wks exposure; spleen, liver, lung], (C) O⁶-HEdG, N1-HEdA, and N6-HEdA adducts [4, 8, or 12 weeks exposure].

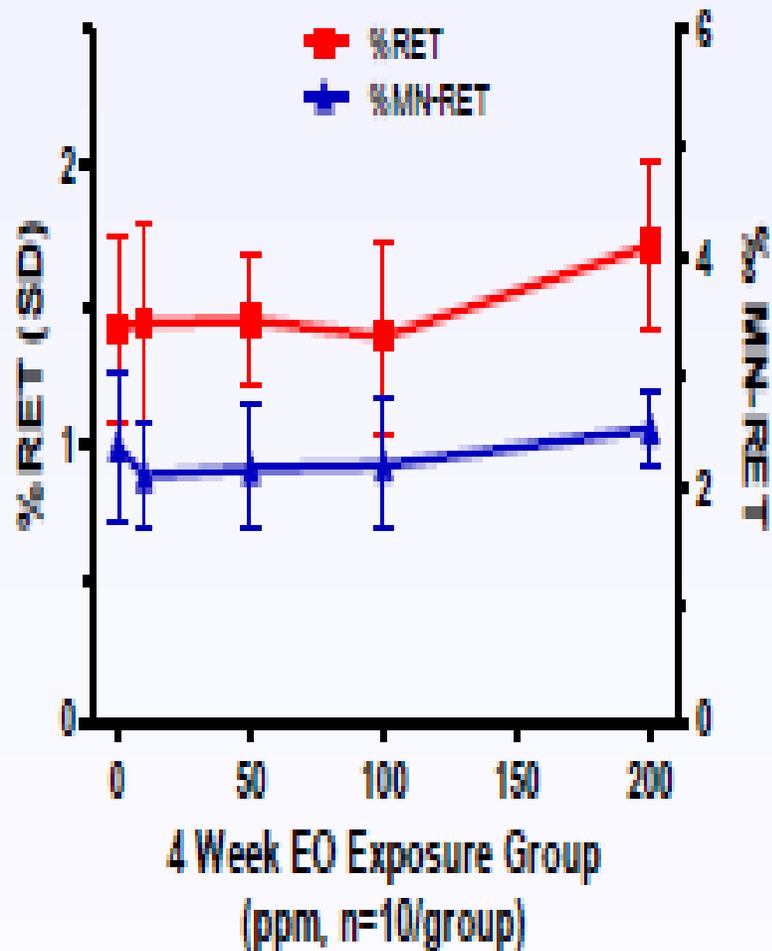


Figure 5. EO Did Not Induce Treatment-Related Increases in Micronuclei or Alterations in Reticulocytes in the Peripheral Blood Following 4 Weeks of Exposure. Peripheral blood was examined by flow cytometry using the MicroFlow® kit and examining ~20,000 RET/animal. Data for each treatment group represent the mean and standard deviation for each endpoint.

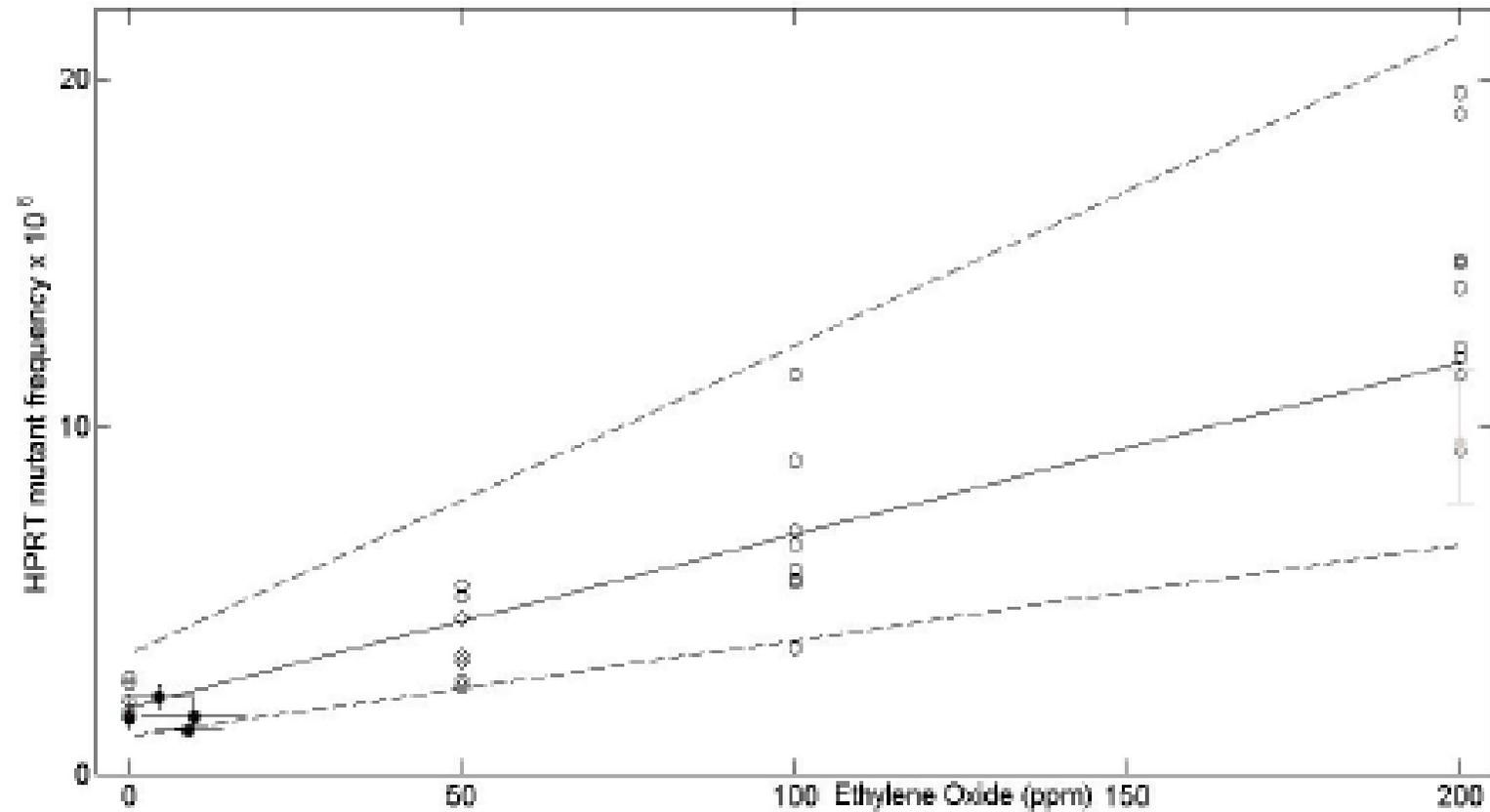


Figure H-1. Induction of *hprt* mutations by EtO (open circles and modeled fit) with data from ethylene (using estimated EtO equivalents) shown (solid circles). Source: [SAB \(2007\)](#), Appendix C (slides 25 and 26); original experiments of [Walker et al. \(1997\)](#).

Table 6. Ratio of observed to NEOTRANS₂-EO model predicted *Hprt* mutant frequencies ($\times 10^{-6}$) for B6C3F1 mice exposed by inhalation to ethylene^a or ethylene oxide

EO(ppm)	Experimentally Observed Mean	Model Predicted ^b	Ratio
0	2	2.1	0.95
0.7 ^a	2.2	2.1	1.05
4.4 ^a	1.1	1.31	0.84
8.6 ^a	1.7	1.57	1.08
50	3.8	4.10	0.93
100	6.8	7.15	0.95
200	14.1	13.3	1.06

^a EO equivalence

^b Predicted mean

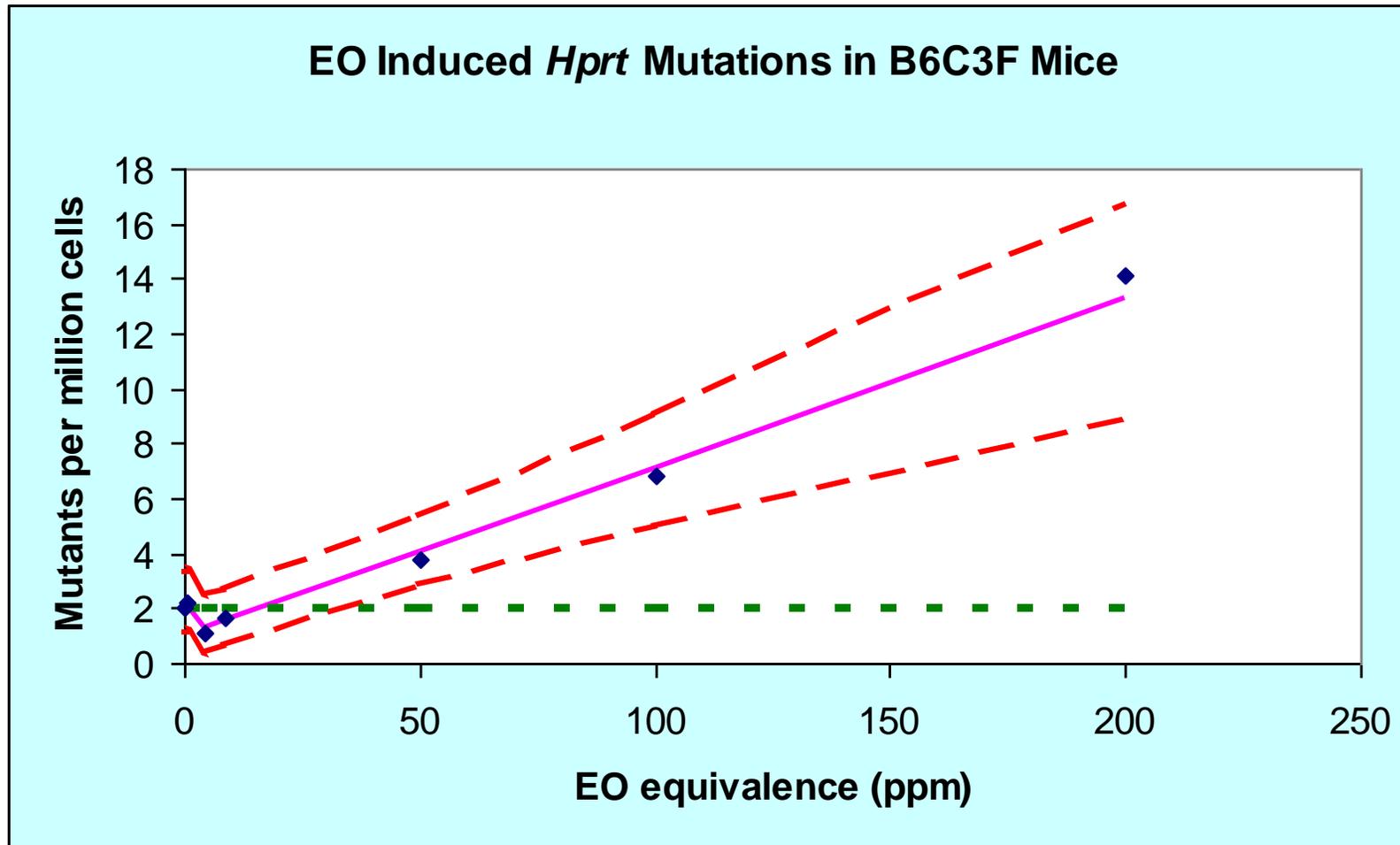


Figure 4. NEOTRANS₂-EO model predicted *Hprt* mutant frequency curve (with 95% confidence intervals) for B6C3F1 mice exposed by inhalation to EO (see also Figure 5). Points = experimentally observed mutant frequency data. Horizontal dashed (green) line represents the spontaneous background mutant frequency.

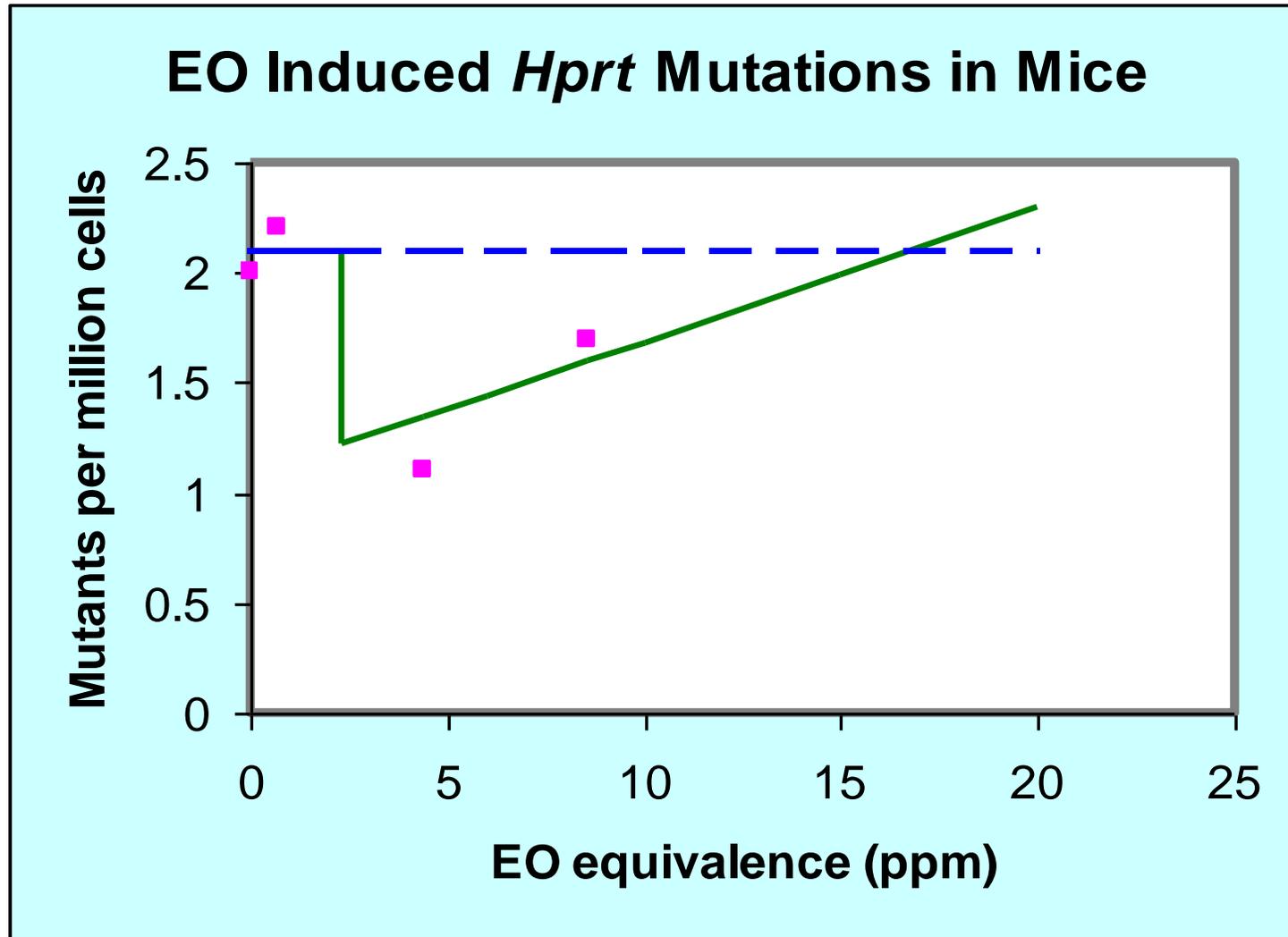


Figure 5. NEOTRANS₂-EO model predicted *Hprt* mutant frequency curve (solid green curve) for B6C3F1 mice exposed by inhalation to EO. Points = experimentally observed mutant frequency data. The first threshold (2 ppm) is for intercellular signaling for protective bystander effects. The threshold for induction of excess mutants by exogenous EO exposure = 17 ppm. Blue dashed line represents the spontaneous background mutant frequency.

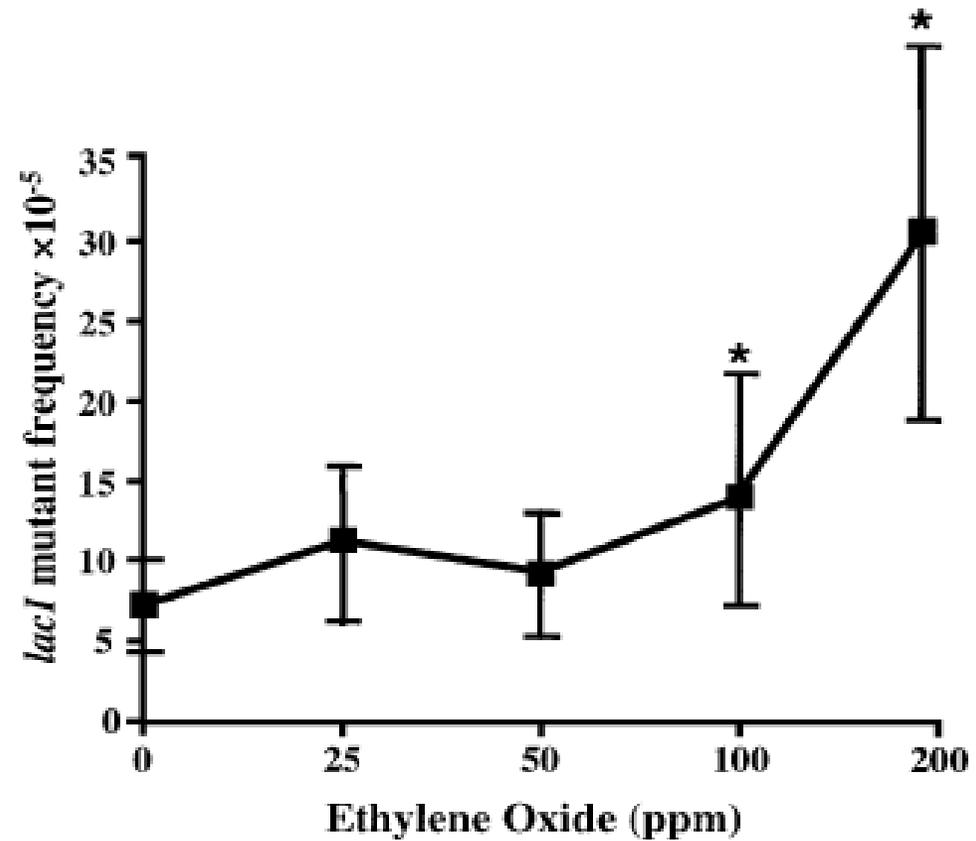


Fig. 1. *LacI* mutant frequency in the bone marrow of B6C3F1 *lacI* transgenic mice at 48 weeks of inhalation exposure to ethylene oxide.

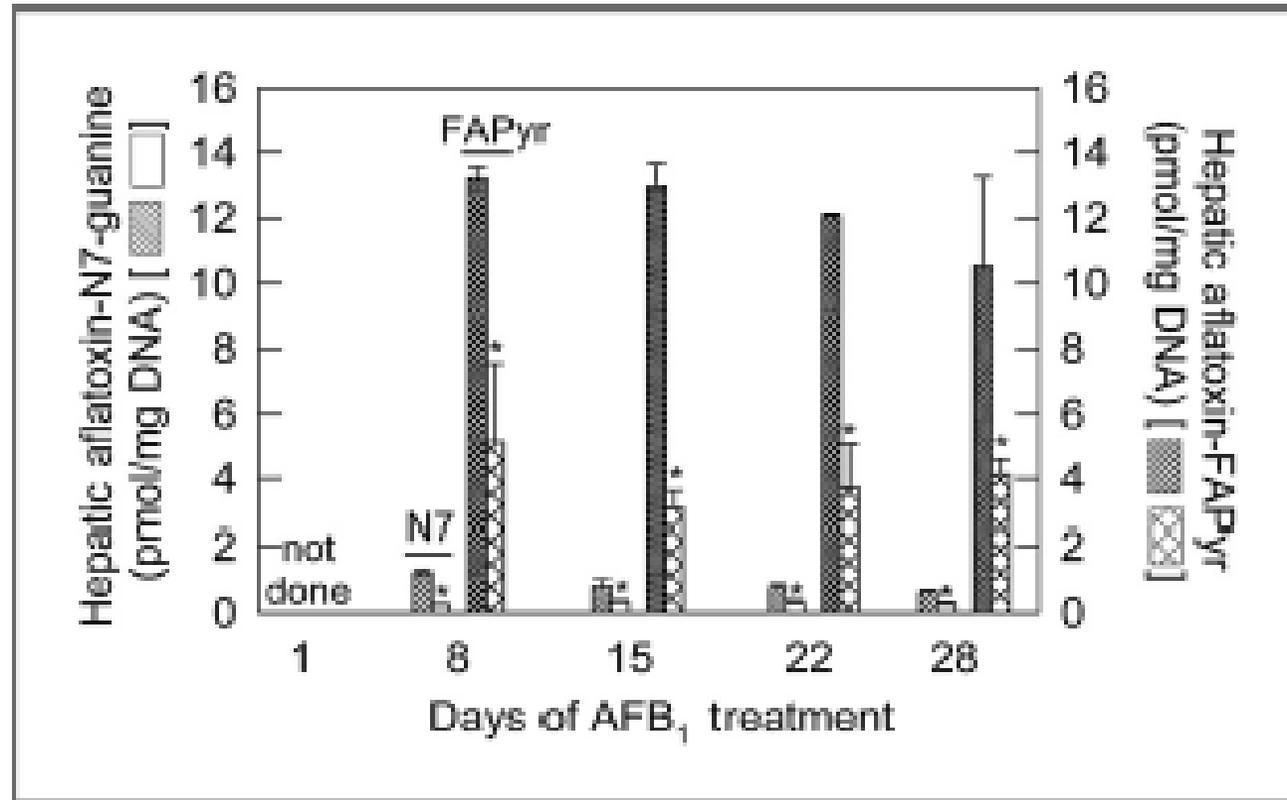


Figure 5. Hepatic levels of aflatoxin-N⁷-guanine (N7) receiving (▨) AFB₁ or (▤) AFB₁ + CDDO-Im and 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxyaflatoxin B₁ (FAPyr) receiving (▩) AFB₁ or (▧) AFB₁ + CDDO-Im in DNA isolated 24 hours after the most recent dose of AFB₁ over a 1 to 4 week dosing period. Values are mean ± SE (n = 3–7).

Complete Protection against Aflatoxin B1- Induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry, Molecular Signature, and Genotoxicity Threshold

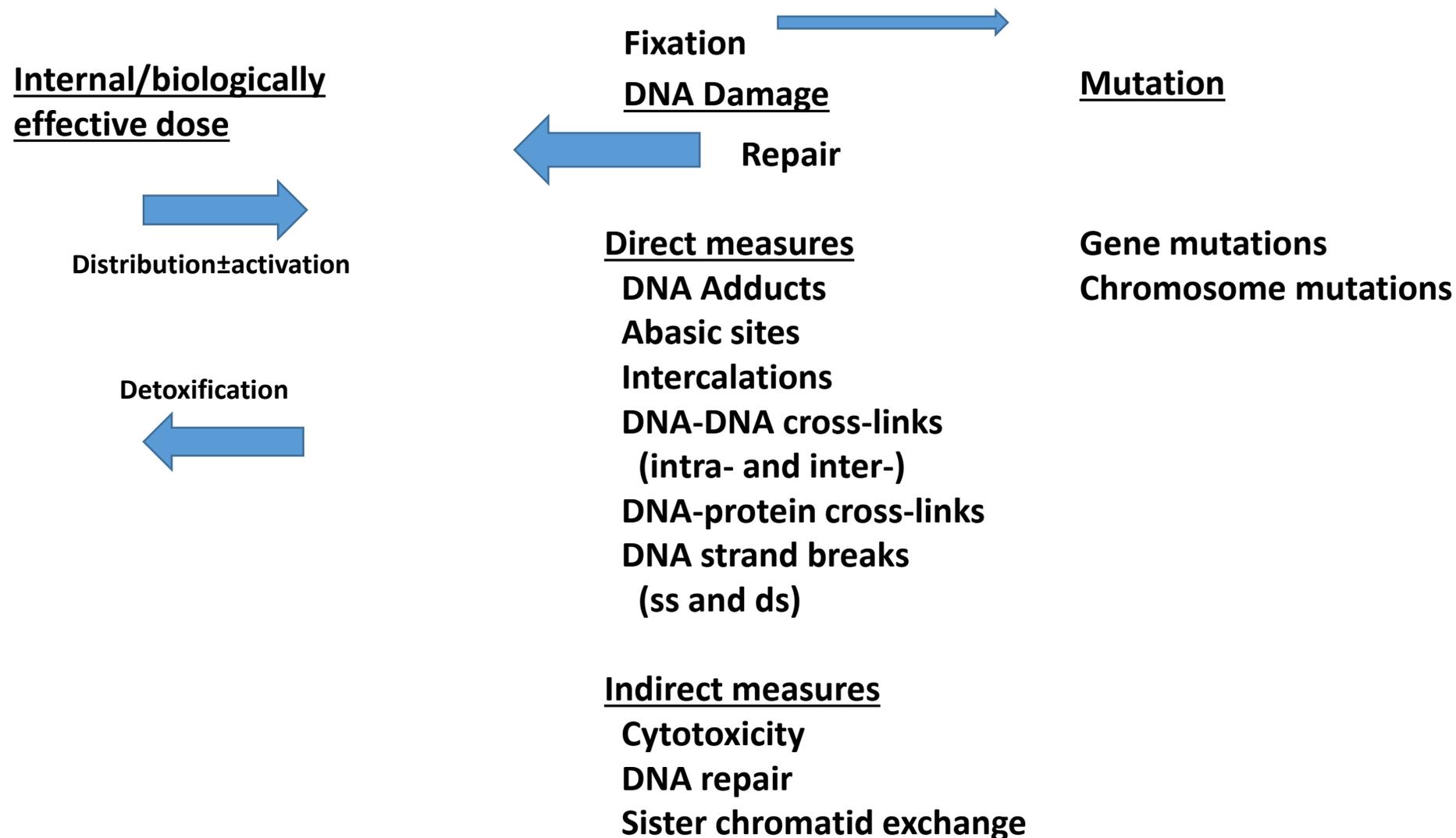
**Natalie M. Johnson¹, Patricia A. Egner¹, Victoria K. Baxter², Michael B. Sporn³, Ryan S. Wible⁴,
Thomas R. Sutter⁴, John D. Groopman¹, Thomas W. Kensler^{1,5}, and Bill D. Roebuck³**

Cancer Res Prev 14(7): 658-665

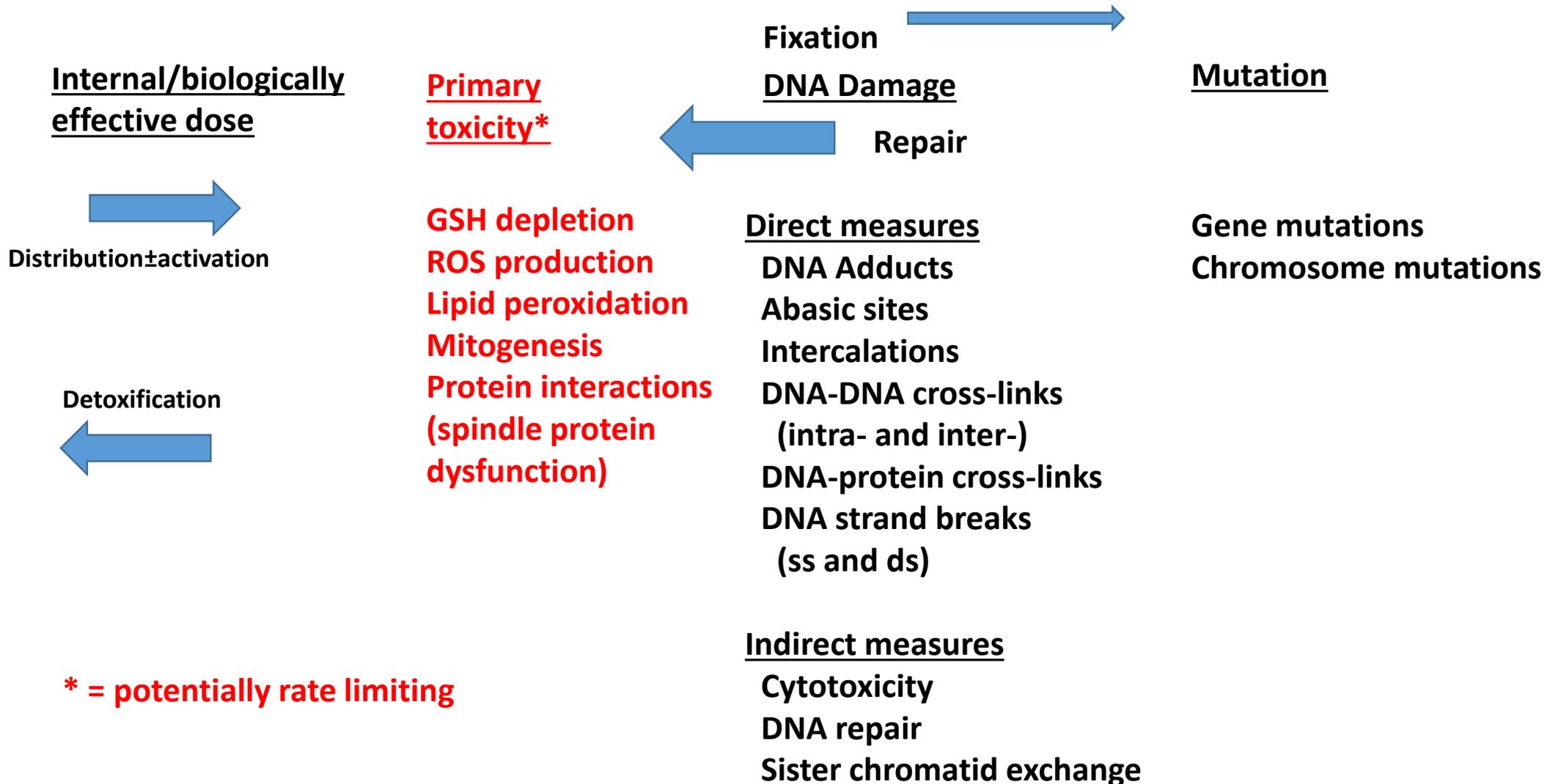
Extrapolation is the most contentious issue in cancer risk assessment. Two basic approaches are used in the extrapolation of observational data from high-dose animal experiments to low-dose human exposure. One of these assumes that there is a threshold dose below which no effect is observed. The other approach assumes that there is no safe dose and that a single molecule is sufficient to increase risk for developing cancer. Our understanding of toxicologic mechanisms has advanced considerably since the linear-no-threshold model was adapted for cancer risk assessment. Knowledge of mechanism of action is critical for informing dose–response relationship below the experimental observable range. Johnson and colleagues (1) have used new technologies in analytical chemistry and molecular biology to characterize downstream biologic events in the exposure disease continuum. **They showed that AFB1 is a classic genotoxic substance in that it binds covalently to DNA and induces mutations. In fact, DNA adduct formation exhibits a characteristic linear dose–response curve over a wide range. But ,further analysis demonstrated a threshold mode of action, with respect to internal dose of active metabolite and hepatocarcinogenesis.** That is, there was substantial adduct formation and DNA damage without having any effect on development of hepatocellular carcinoma. AFB1 is apparently promoting carcinogenesis via a second mode of action downstream to adduct formation related to expression of signature genes involved in hepatocellular carcinogenesis (12) and signaling pathways mediated by nuclear factor erythroid 2-related factor 2 (Nrf2; ref. 13).

Olden and Suryanarayana 2014

Progression of events leading to mutations due to a DNA reactive chemical, i.e. direct, DNA reactive mutagenesis



Progression of events leading to indirectly induced mutations



Ethylene Oxide: An Endogenous Chemical

- Ethylene oxide is a highly reactive chemical that reacts with biomolecules such as proteins and DNA - a property that renders it genotoxic.
- Due to its endogenous production, living organisms are presented with a problem-- they must contend with containing the potential deleterious effects of a highly reactive chemical that they also produce by normal requisite metabolic processes.
- Exposure is inescapable, whether from inhalation, ingestion, or dermal exposure to exogenous sources, from the ethylene gas that is ubiquitous in the natural environment or, critically, as a result of its endogenous production.
- Living organisms cope with the continual presence of ethylene oxide by **maintaining physiological homeostasis** through detoxification.
- **Ethylene oxide's *in vivo* toxicity, including its direct, DNA-reactive genotoxicity, becomes manifest only when exposures result in cellular concentrations that overwhelm these detoxification capacities.**