# Evaluation of Mode of Action for Lung Tumors in Mice Exposed to Ethylene Oxide

Lynne T. Haber[1], Barbara L. Parsons[2], Nigel Moore[3], Bhaskar Gollapudi[4], Mugimane G. Manjanatha[2], Matt LeBaron[4], Martha M. Moore[2], Bruce C. Allen[5]

[1]TERA, Cincinnati, OH, USA [2]Division of Genetic and Molecular Toxicology, National Center for Toxicological Research, US FDA, Jefferson, AR, USA [3]Dow Europe GmbH, Horgen, Switzerland [4]Toxicology & Environmental Research and Consulting, Dow Chemical Company, Midland, MI, USA [5]Bruce Allen Consulting

## Abstract

Ethylene oxide (EO) is a direct acting mutagen and a rodent carcinogen, but it is uncertain whether EO is carcinogenic through a mutagenic mode of action (MOA). To address the MOA for mouse lung tumors, an integrated study was conducted evaluating a variety of biomarkers related to oxidative stress, DNA reactivity/breakage, mutations in a reporter (*cII*) and endogenous (K-ras) gene, and cell proliferation as putative key events, in the target tissue. The dose response and temporality for these key events was assessed in male B6C3F1 mice exposed to 0, 10, 50, 100, or 200 ppm EO (4 weeks) or 0, 100, or 200 ppm EO (8 or 12 weeks) by inhalation (6 hours/day, 5 consecutive days/week). Results indicate that: (1) EO does not appear to cause the lung tumors primarily via its DNA reactivity, since a sustained increase in *cII* MF was not seen at 100 ppm (which produced substantial increases in lung tumors in the mouse NTP study), and the increase at 200 ppm was only weak. (2) Oxidative stress is likely to play a multifaceted role in the tumorigenic process, in light of the absence of an effect on 8-OHdG adducts, and the observed mutation spectra of K-ras codon 12 and *cII*. The role of EO-induced DNA adducts in the etiology of these mutations needs further elucidation. (3) The available data on cell proliferation and K-ras mutations support a MOA that includes effects on intracellular signaling pathways and selection of preexisting K-ras mutant cells, as well as an interplay between oxidative stress and RAS activation. These results suggest a complex multifaceted MOA, potentially involving EO action at multiple steps along the pathway to tumor development.

## Background Study & Design

- Ethylene oxide (EO) is a widely used industrial chemical and classified by IARC as a group I human carcinogen. It can cause gene mutations and chromosome aberrations in vivo and in vitro
- A significant trend of increased benign and malignant lung tumors were seen in a 2-year bioassay in B6C3F1 mice (NTP 1987)
- GSH conjugation is a major pathway of EO metabolism, suggesting a potential role of oxidative stress
- To evaluate the MOA for lung tumors in male B6C3F1 mice, an integrated study was conducted to evaluate a wide range of markers for potential key events related to both a mutagenic MOA (e.g., DNA adducts, mutation in the target tissue), and to an oxidative stress MOA (e.g., 8-oxo-dG, GSH levels)
- Male B6C3F1 mice were exposed to 0, 10, 50, 100, or 200 ppm EO (4 weeks) or 0, 100, or 200 ppm EO (8 or 12 weeks) by inhalation (6 hours/day, 5 consecutive days/week).

## Markers Evaluated

- *cII* (neutral gene) mutant frequency & spectrum in lung
- Micronucleus in peripheral blood
- Comet assay in lung tissue
- Alkylated DNA adducts
  - O6-HEdG, N1-HEdG, N6-HEdG, N7-HEG
- ROS DNA biomarkers
  - 8-OHdG, croton-dG, M1dG
- Lipid peroxidation biomarkers
  - Isoprostanes, prostaglandin f2a
- K-*ras* codon 12 (GGT) mutant fraction in lung:
  - To GTT, GAT, TGT
  - 21/23 EO-induced lung tumors had K-*ras* codon 12 GTT mutations (Hong et al. 2007)
- Cell proliferation (Ki-67)
- GSH-related: GSH, GSSG, HESG, ratios
- Histopathology

See also Manjanatha et al., Poster # 1708; Parsons et al., Poster #1709; Schisler et al., Poster #1707

## Mutagenesis Markers and Interpretation

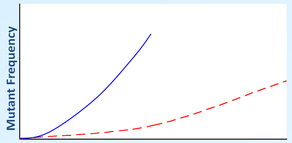
### General Premise for Neutral Gene

- If a chemical causes cancer via direct mutagenesis, there are two basic predictions.
  - Mutations will be observed in relatively short time periods following treatment and prior to the observation of tumors
  - Mutations will be induced at doses lower than or equal to those required to form tumors

### Temporality—Evaluate time-to-mutation

**Predictions**

Mutagenic carcinogens would be expected to show a positive mutation response after relatively short treatment periods (blue line)




Nonmutagenic carcinogens would be expected either not show an increased mutation response, or require a long chronic treatment for a positive response (red line)

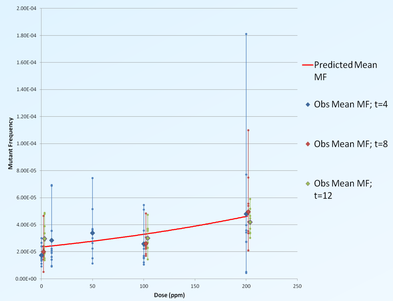
**Table 1. Comparison of *cII* MFs in the lung of BB mice and Lung Tumor Response**

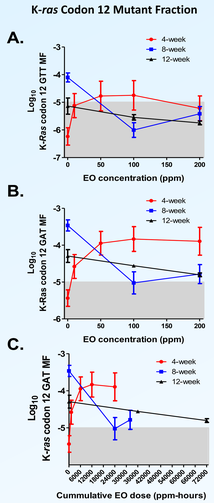
| | Average *cII* MFs in the Lung of BB Mice[a] | | | Tumor[b] Response (%) (NTP, 1987) |
|---|---|---|---|---|
| Concentration (ppm) | 4 WK- MF | 8 WK- MF | 12 WK- MF | |
| 0 | 17.4 ± 2.2[d] | 20.0 ± 3.4 | 29.6 ± 4.1 | 11/50 (22) |
| 10 | 28.5 ± 7.1[†] | NT[c] | NT | NT |
| 50 | 33.8 ± 5.8[†] | NT | NT | 19/50 (38) |
| 100 | 25.9 ± 5.1 | 26.1 ± 2.8 | 30.0 ± 2.9 | 26/50 (52)[‡] |
| 200 | 47.9 ± 16.2[†] | 49.6 ± 8.5* | 41.9 ± 2.8* | NT |

a. See Manjanatha et al., Poster # 1708; each value is the mean of 10 mice ± SEM
b. Alveolar/bronchiolar adenoma or carcinoma
c. NT, not tested
d. In a no statistical significance of trend (NOSTASOT) evaluation of the 4-week data, only the 200 ppm data point was significant.
* Significantly different from the concurrent control MF at p≤ 0.05 in pairwise comparisons using Chi square; data were log-transformed due to the increasing SD of the MF with increasing MF.
†Significantly different p≤ 0.05 from concurrent control in a pairwise comparison using a beta distribution and Chi square.
‡Significantly different from control

### Supplemental Statistical Analysis

- Used beta regression techniques to determine the presence of trends with respect to concentration and duration of exposure
- When the variance was allowed to be nonconstant, the analysis suggested that there were trends of increasing mean MF with increasing concentration, at all three durations.
- Treating concentration and duration as continuous explanatory variables, trends were quantified using a variety of models.
- In the best model (judged by AIC and BIC), mean MF was modeled as a function of EO concentration, but not duration of exposure




### Issues and Challenges

- How do we compare MF and tumor response
  - Biological significance vs. statistical significance
  - What is a biologically meaningful increase in MF? (tie to background cancer incidence?)
  - Comparing curves of probability of effect avoids definition of "adverse increase in MF"
- Challenges also in considering the increasing background MF, large variability at high concentration

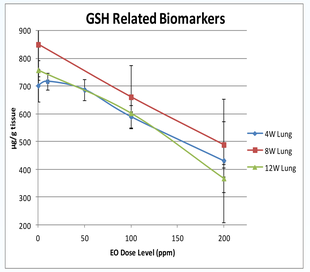
### Conclusion for Neutral Gene

- Conclusion: Clear and consistent increase in MF only at 200 ppm. Although 100 ppm is lower than might be expected at 4 weeks, it is clearly negative at later time points in pairwise comparisons.
- Supplemental statistical analyses suggest no change across duration.
- Expect that response would increase with time for strong mutagens, which is not seen here.
- EO is not a strong in vivo mutagen in the target tissue.

## Weight of Evidence for Potential Key Events

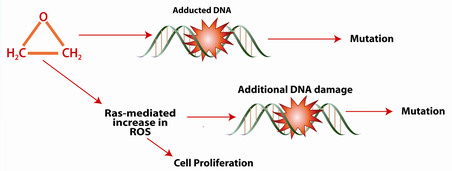
| Evidence for | Evidence against |
|---|---|
| **Reactive oxygen species (ROS) – minimal effect** | |
| 50% decrease in GSH @ 200 ppm, all durations | GSH is in excess in cell; do not expect effect on ROS until decreased by ~60-70% (Albertini & Sweeney, 2007) |
| Trend in decreased GSH, but decrease at 50 and 100 ppm is minimal | Not solely compensation; Brown et al. (1998) found 50% decrease in lung GSH at 100 ppm after a 4-hour exposure |
| Increased lipid peroxidation in lung but not spleen or liver – based on some of the isoprostane markers<br>--Increase is concentration related, apparent at 100 ppm | Not all isoprostane markers are affected |
| **ROS leading to 8-OHdG (potentially leading to G→T mutation) – suggestive of indirect effect** | |
| Concentration related increase in 8-OHdG | Increase in 8-OHdG is small |
| Increase in 8-OHdG stronger in lung than liver<br>(but comparable in lung and spleen) | Incidence of K-*ras* codon 12 GGT→TGT mutations did not exceed level of detection - same mutation as the GGT→GTT |
| | K-*ras* codon 12 GGT→GTT MF sig *decreased* at wks 8 & 12 – suggestion of selective elimination |
| K-*ras* codon 12 GGT→GTT mutation in 21/23 EO-induced lung tumors | K-*ras* codon 12 GGT→GTT MF shows inverse correlation to *cII* MF |
| *cII* mutation spectrum consistent with effect on 8OHdG | No sustained stat sig increase in *cII* gene mutations at 100 ppm |

| Evidence for | Evidence against |
|---|---|
| **Direct DNA alkylation adducts- consistent with weak mutagen** | |
| Substantial concentration-related increase in N7 adducts down to 10 ppm, with somewhat higher levels in lung than liver and spleen | Adducts in all tissues – not specific to tumor target |
| *cII* mutation spectrum consistent with alkylation adducts leading to AP sites | N7-HEG has weak mutagenic activity. E.g., no increase in abasic sites in rats exposed to 100 ppm for 4 weeks (Rusyn et al., 2005) |
| Trend of increased mutations across time | No sustained inc in *cII* gene mutations at 100 ppm |
| **Mitogenesis – early selective amplification of pre-existing K-*ras* mutation** | |
| Inc K-*ras* codon 12 GGT→GAT – amplification of most common spontaneous mutation in mouse lung tumors at 4 weeks | |
| No single type of DNA adduct correlates with K-*ras* codon 12 GAT or GTT mutations | Observed profile of induced mutation could be due to combination of adducts |
| K-*ras* codon 12 GGT→GAT and GTT mutations correlated within individual mice | No *generalized mitogenesis*, based on histopathology and Ki-67 data |
| **Clastogenicity – No evidence for downstream events** | |
| Strong and consistent evidence for clastogenicity in vitro and in vivo | |
| Increased chromosome aberrations in exposed workers | |
| Increased strand breaks (comet assay) in current assay (abstract #1707) at 4 wks at ≥50 ppm | No increase in micronuclei in peripheral blood in current assay |
| Inc MN in type II cells and Clara cells in mice exposed to 630 mg/m³ (350 ppm) for 4 hr - so MN after single exposure with AUC less than that from 8 hr to 200 ppm (Lindberg et al. 2010) | (Current assay did not directly evaluate clastogenesis in target tissue – *cII* only detects point mutations) |
| Conc- and duration-related inc in chrom aberrations and translocations in lymphocytes in range of bioassay conc (Donner et al. 2009). Stat sig down to 25 ppm at 48 wks. | |




## Evidence Related to Earlier Hypothesized MOAs

- Kirman et al. (2004) suggested EO-induced leukemia results from formation of 2 DNA adducts in rapid succession, representing ends of translocation or deletion, followed by translocation or deletion affecting critical gene for cell division.
  - No general stimulation of mitogenesis (based on histopathology or Ki-67); instead, selective stimulation, then elimination of K-ras mutant cells.
  - A limitation of the current work is that although micronuclei in blood and single strand breaks were evaluated, none of the measures in the target tissue directly measured chromosome aberrations.
  - However, chromosome aberration is associated with EO-related leukemia, but not lung tumors.
- Sweeney et al. (2009) proposed that propylene oxide (PO)-induced nasal tumors result from sustained, severe target tissue GSH depletion as an initial key event, with GSH depletion and increased cell proliferation augmenting genotoxicity resulting from direct DNA reactivity and from non-DNA reactive genotoxicity of PO.
  - At tumorigenic doses of EO, GSH depletion is much lower than observed for PO and there is no evidence of inflammation in current assay or NTP bioassay.




## Conclusions

- EO MOA is complex, and does not follow classical patterns, e.g., for mutation, oxidative stress, or cytotoxicity/compensatory cell proliferation
- A role for direct gene mutation cannot be ruled out, but EO is a weak mutagen in the target tissue
- Many effects appear to have a stronger response at 4 weeks than later time points
  - Some early changes may have been missed, but effect of GSH depletion at 100 ppm on ROS even at early time points is unclear
- Evidence for selective amplification and then selective elimination of K-ras mutant cells
- Observation of gene mutations in the target tissue in a neutral gene or cancer-related gene is not sufficient to show a mutagenic MOA; need to consider dose-response and exposure duration-response.
- The MOA for EO is still uncertain, but it does not appear to be driven by direct mutagenesis. ROS and downstream effects on cell signaling through K-ras have been hypothesized (abstract #1709), but it is unclear whether ROS generation is sufficient to be a driver.