The Key Characteristics of Carcinogens as an Organizing Principle for Mechanistic Evidence: Ethylene Oxide as a Case Study

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Structured Overview

Background – Ethylene Oxide (EtO) Assessment

In 2016, the U.S. Environmental Protection Agency's Integrated Risk Information System Program finalized a cancer assessment of ethylene oxide (EtO), characterizing it as "carcinogenic to humans" following inhalation exposure. EtO induces lymphoid and breast cancers in both humans and rodents, as well as other tumors in rats and mice (U.S. EPA, 2016).

Role of Mechanistic Data

While strong epidemiological evidence was instrumental in the human health hazard characterization process, evaluation of the animal and mechanistic data was also critically important (Jinot et al., 2018). Core concepts from the key characteristics of carcinogens (KCCs) (Smith et al., 2016), a pragmatic means of categorizing and evaluating the weight of evidence for mechanisms of carcinogenesis, were adopted in the organization of the mechanistic data summary sections supporting the mode of action analysis.

Evolving Approach to Evaluating Mechanistic Data

In subsequent work, the mechanistic evidence identified in the comprehensive literature search included in the IRIS assessment has been further reviewed and organized in a systematic manner using the KCCs as an organizing principle; this information was reviewed using a weight of evidence approach and integrated into adverse outcome pathways.

Conclusions Based Upon Key Characteristics of Carcinogens (KCCs)

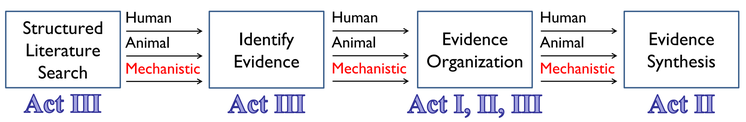
Strong and consistent evidence indicates that EtO is both electrophilic and mutagenic, representing two of the 10 KCCs; conversely, evidence for oxidative stress, another KCC, was neither strong nor consistent. Evidence of coherence in genetic or genomic damage in similar tissues across rodents and humans provides further support, linking relevant associations across data streams. The evaluation and discussion of cancer mechanisms was facilitated by using the KCCs as a central organizing principle to evaluate mechanistic data.

Challenges and Future Directions

One significant challenge in the systematic evaluation of cancer mechanisms was a paucity of mechanistic data identified from the EtO assessment literature search to evaluate 7/10 of the KCCs; specific supplemental literature searches using a digital content management system (i.e., Health Assessment Workplace Collaborative, or HAWC) have since been performed to locate published information pertinent to each KCC, based upon the methods recently described by Guyton et al. (2018). KCCs and Evidence Profile Tables (EPTs) are used in the structured evaluation of mechanistic data relevant to cancer hazard assessment, along with the database assembled and published in the EtO Assessment (U.S. EPA 2016), and described subsequently by Jinot et al. (2018).

Evolving Mechanistic Data Evaluation – in Three Acts

Hazard Identification Process (Adapted from NRC 2014, Review of EPA's IRIS Process)



Act I – US EPA IRIS Assessment of EtO (U.S. EPA, 2016)

- Mechanistic evidence sections organized by KCCs
- Mechanistic evidence separately synthesized according to 2 mode of actions (MOAs)

Act II – Discussion of Cancer Mechanisms (Jinot et al., 2018)

- Mechanistic evidence discussion utilized structured tables for high-level summary of primary observations by data stream and key event
- Mechanistic evidence synthesis of 2 MOAs followed structured summary

Act III – KCCs to Facilitate Structured Evaluation (*ongoing…*)

- Structured literature searches designed specifically to capture KCCs
- Evidence organization and summary of observations facilitated by EPTs

U.S. Environmental Protection Agency
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Act I – US EPA IRIS EtO Assessment

Relevant KCC	Possible Key Event
1) Is Electrophilic or Can Be Metabolically Activated	Protein adducts
	DNA adducts
2) Is Genotoxic	Point mutations in reporter genes or surrogate markers
	Point mutations in proto-oncogenes or tumor suppressor genes
	Chromosomal effects
5) Induces Oxidative Stress	ROS or lipid peroxidation products
	DNA oxidation
	Glutathione species levels

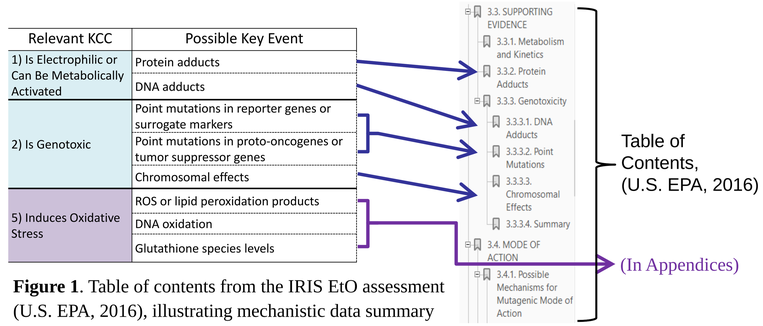

Figure 1. Table of contents from the IRIS EtO assessment (U.S. EPA, 2016), illustrating mechanistic data summary and organization by KCC, supporting MOA analysis.

Act II – Discussion of Cancer Mechanisms

Directly Mutagenic MOA

Relevant KCC	Possible Key Event	Weight-of-Evidence: Exposed Humans	Weight-of-Evidence: Experimental Systems (*In Vivo*)	Evidence Integration and Conclusions
1) Is Electrophilic or Can Be Metabolically Activated	**DNA adducts**	**N7-HEG** adducts were non-significantly elevated in white blood cells in two studies.	**N7-HEG** adducts increased in multiple rat and mouse tissues following repeated exposures to ≥ 3 ppm. **O^6-HEG** and **N3-HEA** adducts were also detected in rats exposed to 300 ppm; **O^6-HEG** and **N1-** and **N^6-HEA** adducts increased in an exposure-related manner in mice exposed to 100 or 200 ppm.	EtO is a direct-acting agent capable of alkylating DNA, proteins and non-protein sulfhydryls (e.g. GSH). **Strong and consistent evidence** demonstrating protein adduction in humans and rodents, as well as DNA adduction in rodents, along with **weak but not inconsistent evidence** from the limited human database of DNA adduct studies.
	Protein adducts	Exposure-response relationships have been reported with **hemoglobin adducts** in several studies; such adducts can be used as biomarkers of recent human exposure to EtO.	**Hemoglobin adducts** are linearly correlated with exposures up to at least 33 ppm in rats and mice; **adduction increased** at exposures ≥ 100 ppm, consistent with decreased GSH availability for detoxification. EtO-conjugated glutathione (**HESG**) increased in an exposure-related manner in mouse lungs following subchronic exposures ≤ 200 ppm.	
2) Is Genotoxic	**Genetic mutations**	***HPRT*** mutant frequency was significantly increased in peripheral blood leukocytes in one study following high exposures.	***Hprt*** or ***Lacl*** mutation incidences increased in several tissues in rats and mice, and dominant lethal effects were observed in germ cells.	**Strong and consistent evidence** supporting the induction of point mutations and chromosomal effects in rodent tissues, and the induction of chromosomal effects in humans. Most tumor mutations in genes regulating repair, proliferation, or survival in EtO-exposed mice occurred at purine nucleotides, consistent with evidence of EtO DNA adduction.
	Mutations in DNA repair, proliferation, or survival genes	*No evidence available*	***Kras*** mutation incidence was higher and the mutational spectra differed in lung and Harderian gland tumors of EtO-exposed mice, compared with tumors from control mice. The ***Trp53*** and ***Hras*** mutational spectra were likewise different in mammary gland carcinomas from EtO-exposed mice; these genes were frequently mutated together, while ***Trp53*** mutations and protein expression were induced in an exposure-dependent manner.	
	Chromosomal effects	**Chromosome aberrations** and **sister chromatid exchanges** were elevated in peripheral blood leukocytes from populations with prolonged and/or intense exposures.	**Sister chromatid exchanges** were induced in lymphohematopoietic tissues in non-human primates, rats, and rabbits; **chromosome aberration** incidence increased in similar tissues in mice, as well as in germ cells.	

Oxidative Stress MOA

Relevant KCC	Possible Key Event	Weight-of-evidence: Exposed Humans	Weight-of-evidence: Experimental Systems (*In Vivo*)	Evidence Integration and Conclusions
5) Induces Oxidative Stress	**↑ ROS, lipids oxidation or lipid-dNTP products**	*No evidence available*	**Croton-dG** increased in mouse lungs in an exposure-related manner with subchronic exposures ≤ 200 ppm; other lipid-nucleotide adduct species evaluated were unaffected.	**No evidence** supports DNA or GSH oxidation following subchronic EtO exposure. The decreased GSH and GSSG levels likely resulted from EtO-GSH conjugation, and not oxidation. No direct measures of ROS were reported, and **limited evidence** supports increased levels of a single oxidized lipid-DNA adduct (CrotondG).
	↑ 8-OHdG levels or direct DNA oxidation	In human lung epithelial cells, keratinocytes and PBLs exposed *in vitro*, **oxidative DNA damage** (Fpg-dependent comet assay) was not increased.	**8-OHdG** levels did not increase in mouse lungs following subchronic exposures ≤ 200 ppm.	
	↑ GSH oxidation (↓GSH/GSSG)	*No evidence available*	Both **GSH** and **GSSG** decreased in the lungs of mice an exposure-related manner following subchronic exposures ≤ 200 ppm; however, the **GSH:GSSG ratio** and **total glutathione content** were not affected.	

Table 1. Summarized support for mechanistic key events from the IRIS EtO assessment (Adapted from U.S. EPA, 2016 and Jinot et al., 2018), organized by KCC, key event, MOA and evidence stream.

Act III – KCCs in Lit. Searching and Evidence Identification

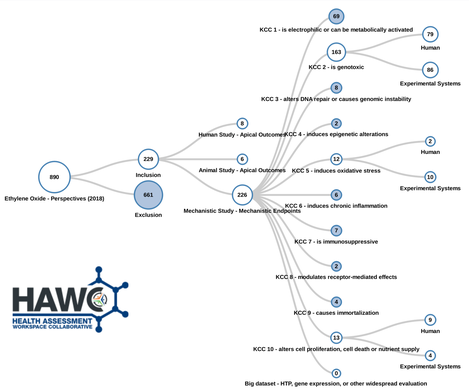

Figure 2. LitFlow tagtree from HAWC. Independent mechanistic literature search strings were constructed (Guyton et al., 2018), for KCCs1-3, KCC4, KCC5, KCCs6-7, KCC8, and KCCs9-10. Circles indicate number of studies categorized following title/abstract screen; a significant number of identified studies were excluded for lack of relevance, and there was a paucity of data relevant to many KCCs.

Act III – KCCs/AOPs/MOAs in Evidence Organization

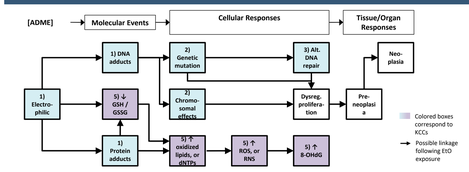

Figure 3. Plausible pathways relating key events (including relation to KCCs) leading from exposure to carcinogenesis, relevant to genotoxicity and oxidative stress activity.

Outcomes (KCCs and key events)		Studies and interpretation	Factors that increase strength	Factors that decrease strength	Summary of findings	Within stream evidence judgement	Overall conclusion
Directly Mutagenic MOA							⊕⊕⊕ STRONG – Mutagenic MOA Based upon: • Strong and consistent evidence of direct alkylating activity, genotoxicity and mutagenicity in humans and a variety of experimental systems • Evidence of temporality and an exposure-response gradient between • Widespread coherence across outcomes and species • Biological plausibility between supported key events and carcinogenesis Relevance to humans: • Strong evidence that the key precursor events are anticipated to occur in humans and progress to tumors, including human evidence of chromosome damage, such as chromosomal aberrations, SCEs, and micronuclei.
1) Electrophilic	DNA adducts	*Many* – Table 3-6; e.g. Zhang et al., 2015; Marsden et al., 2009, 2007; Young et al., 2007; Ruoyn et al., 2005, etc.	• Exposure-response gradient • Coherence (species) • Pro-mutagenic	• Few studies available (human) • Effect size (human)	⊕⊕⊕ STRONG, consistent and coherent in rodent studies	⊕⊕⊕ STRONG and consistent evidence of protein adduction in humans and rodents, and DNA adduction in rodents; while human evidence of DNA adducts are limited.	
	Protein adducts	*Many* – Section 3.3.2; e.g. IARC 2008; Walker et al, 1993, 1992, etc.	• Exposure-response gradient • Coherence (species) • Coherence (molecular targets)		⊕⊕⊕ STRONG, consistent and coherent		
2) Genotoxic	Genetic mutations	*Many* – Table 3-6; e.g Major et al., 2001; Walker et al., 2000; Tates et al., 1999, etc.	• Coherence (species)	• Few studies available (human) • Effect size (human)	⊕⊕⊕ STRONG, consistent and coherent in rodent studies	⊕⊕⊕ STRONG and consistent evidence for induction of point mutations and chromosomal effects in rodent tissues, consistent with strong and consistent evidence of chromosomal effects in humans.	
	Mutations in DNA repair, proliferation, or survival genes	Hong et al., 2007; Houle et a., 2006 (from NTP, 1987); Parsons et al., 2013	• Coherence (tissues) • Mutational fingerprint • Biological plausibility (*ras*, *Trp53* genes)	• No data (human) • Coherence (temporal)	⊕⊕○ MODERATE, coherence in multiple tissues from rodent studies, and plausible fingerprint		
	Chromosomal effects	*Many* – Tables 3-7 and 3-8; e.g. Donner et al., 2010; Major et al., 1996, etc.	• Coherence (species, tissues) • Exposure-response gradient	• Few studies available for some endpoints (e.g. micronuclei incidence)	⊕⊕⊕ STRONG, consistent and coherent		
Oxidative Stress MOA							
5) Oxidative stress	ROS, lipid oxidation	Zhang et al., 2015		• Few studies available • Effect size	○○○ INADEQUATE	⊕○○ SLIGHT evidence for increased levels of an oxidized lipid-DNA adduct, and no evidence of direct DNA or GSH oxidation, or ROS generation.	
	DNA oxidation	Zhang et al., 2015; Nagy et al., 2013	• Exposure-response gradient in CrotondG (mice)	• Few studies available • Effect size (human) • Coherence (within, between species)	⊕○○ SLIGHT Increases in one, but not other markers, in one study/sex/species		
	GSH oxidation	Zhang et al., 2015; Brown et al., 1998		• Few studies available • Effect size • Changes attributed to alkylation	○○○ INADEQUATE		

Table 2. Preliminary EtO cancer mechanism EPT, summarizing conclusions on the strength of available evidence relevant to KCCs and key events, for both mutagenic and oxidative stress MOAs (note that glutathione can be oxidized, alkylated, and/or depleted via metabolism).

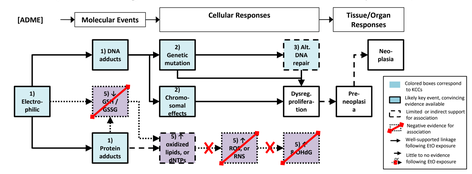

Figure 4. Illustration of observations summarized in EPT above, overlaid on relevant mechanistic pathways, as part of evidentiary support for MOA analyses and cancer synthesis.

Observations and Future Directions

- KCCs provide a topical basis for focused, transparent and reproducible literature searches
- Summary tables and EPTs are useful as structural tools for organizing large volumes of mechanistic data and highlighting results of evaluation by subject-matter experts in what could be a transparent, reproducible, and understandable manner
- Data relevance, organization, weight of evidence, and pathway impact determinations are currently highly expert judgement-driven, and subjective; more work is needed to develop objective, pragmatic, and reproducible *a priori* criteria

Selected References

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