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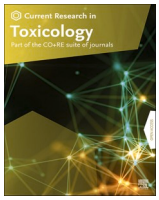

Review Article

Systematic evaluation of the evidence base on methyl *tert*-butyl ether supporting a lack of concern for carcinogenic hazard in humans based on animal cancer studies and mechanistic data

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ABSTRACT

Methyl *tert*-butyl ether (MTBE) is a high-octane fuel component that helps gasoline burn cleaner and reduces automobile emissions. In 1999, the International Agency for Research on Cancer (IARC) categorized MTBE as "not classifiable" regarding human carcinogenicity. Since then, additional studies have been published that substantially added to the evidence base to examine the carcinogenic potential of MTBE in humans. A systematic literature search and review was conducted to identify mechanistic data, as well as studies investigating cancer in MTBE-exposed humans and experimental animals. Critical appraisal was performed for relevant studies with mechanistic data organized and evaluated within Key Characteristics of Carcinogens (KCCs). Three standard animal cancer bioassays showed a low incidence of hepatocellular adenomas in female mice (inhalation exposure), with renal adenomas/carcinoma (inhalation) and brain tumors (drinking water) in male rats exposed to high concentrations of MTBE. Evidence extracted from the literature demonstrate that the mechanism of male rat renal tumors does not operate in humans. Review of the strength of mechanistic data was based on activity, relevancy, and reliability, with information-dense KCC2—is genotoxic, and KCC10—alters cell proliferation, cell death, and nutrient supply, together supporting that MTBE is unlikely to be a carcinogenic hazard to humans.

Introduction

Methyl *tert*-butyl ether (MTBE) (CASRN 1634–04-4) is a volatile organic compound first added to gasoline in the mid-1980s, with peak usage in the United States (US) in the 1990s. MTBE was incorporated into gasoline to meet the US Environmental Protection Agency's (EPA) Amendment to the Clean Air Act requirement that reformulated gasoline contains at least 2% oxygen by weight. In 1999, several states in the US began banning the use of MTBE based on its low odor threshold (15 ppb) and presence in groundwater from underground storage tank (UST) leakage rather than because of specific health-related concerns. (EIA, 2020; EPA BRP, 1999). Following the US Energy Policy Acts of 2005 and 2007 requiring the use of 10% ethanol, MTBE was phased out as a gasoline oxygenate in the US (EPA, 2007; Bogen and Heilman, 2015). MTBE is still manufactured globally and used as a gasoline fuel additive in many countries in part due to its function as an oxygenate that reduces both tailpipe emissions and criteria pollutants (EIA, 2020, ATSDR 2023). Human exposure to MTBE may still occur in occupationally exposed workers, and in the general population via inhalation, oral, or dermal exposure from contaminated water sources, although this has greatly diminished since the adoption of enhanced UST standards in most countries (ATSDR, 2023; EPA, 2015). As such, extensive human health effect studies on MTBE have been conducted over the last 30 years.

Several regulatory and non-regulatory assessments on MTBE's human health effects have been published, including an evaluation by the World Health Organization (WHO) International Programme on Chemical Safety (IPCS) (1998), Health Canada, (2006), California EPA

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Public Health Goal (1999), and, most recently, the Toxicology Profile by the Agency for Toxic Substances and Disease Registry (ATSDR, 2023) where cancer outcomes are discussed. The International Agency for Research on Cancer (IARC) evaluated human cancer hazard associated with MTBE exposure in 1999 and concluded that MTBE was *"not classifiable as to its carcinogenicity for humans (Group 3)"* (IARC, 1999). This conclusion was based on the lack of available human cancer studies, three experimental cancer studies of which two inhalation studies (in rats and mice) were considered (Bird et al., 1997) and one oral gavage cancer study (in rats) was noted to have significant limitations (Belpoggi et al., 1995;1997). Also, it was noted that MTBE lacked genotoxicity along with documentation that the male rat specific kidney tumors identified was through a mechanism that does not operate in humans (Capen et al., 1999). IARC is scheduled to reevaluate MTBE in 2025, along with other oxygenates and automotive gasoline. Since 1999, new studies have been published to evaluate MTBE's toxicity and carcinogenic potential in experimental animal models when exposed via drinking water; a 90-day subchronic toxicity study and a standard cancer bioassay (Bermudez et al., 2012; Dodd et al., 2013). Also, since IARC's initial evaluation there has been a significant body of mechanistic data, including publication of mutagenic and genotoxic studies, along with studies that include mechanistic endpoints organized across the Key Characteristics of Carcinogens (KCCs). Currently, IARC uses the KCCs to organize and evaluate mechanistic data to evaluate the potential of substances to cause cancer in humans (IARC Preamble, 2019). The KCCs are a series of ten characteristics used to evaluate a chemicals' potential carcinogenic activity through multiple, or a single mechanism. These characteristics were initially published by Smith et al. (2016) but have been used by IARC in the classification of potential human carcinogens, as updated in the current preamble (IARC Preamble, 2019). Organization of mechanistic data within these KCCs is now utilized by the National Toxicology Program (NTP) Report on Carcinogens (RoC) (NTP, 2018) and California EPA (2021) in hazard assessments and also used to consider mechanistic activity associated with key events in carcinogenic adverse outcome pathways and modes of action for selected carcinogens (Meek et al., 2014; Meek and Wikoff, 2023).

The objective of this assessment is to systematically review the entire body of evidence for MTBE as it pertains to carcinogenic potential in humans. While comprehensive reviews have previously evaluated the potential carcinogenic activity of MTBE (IARC, 1999; Bogen and Heilman, 2015; Bus et al., 2022; ATSDR, 2023), a complete integration of all findings, including the robust mechanistic evidence base organized

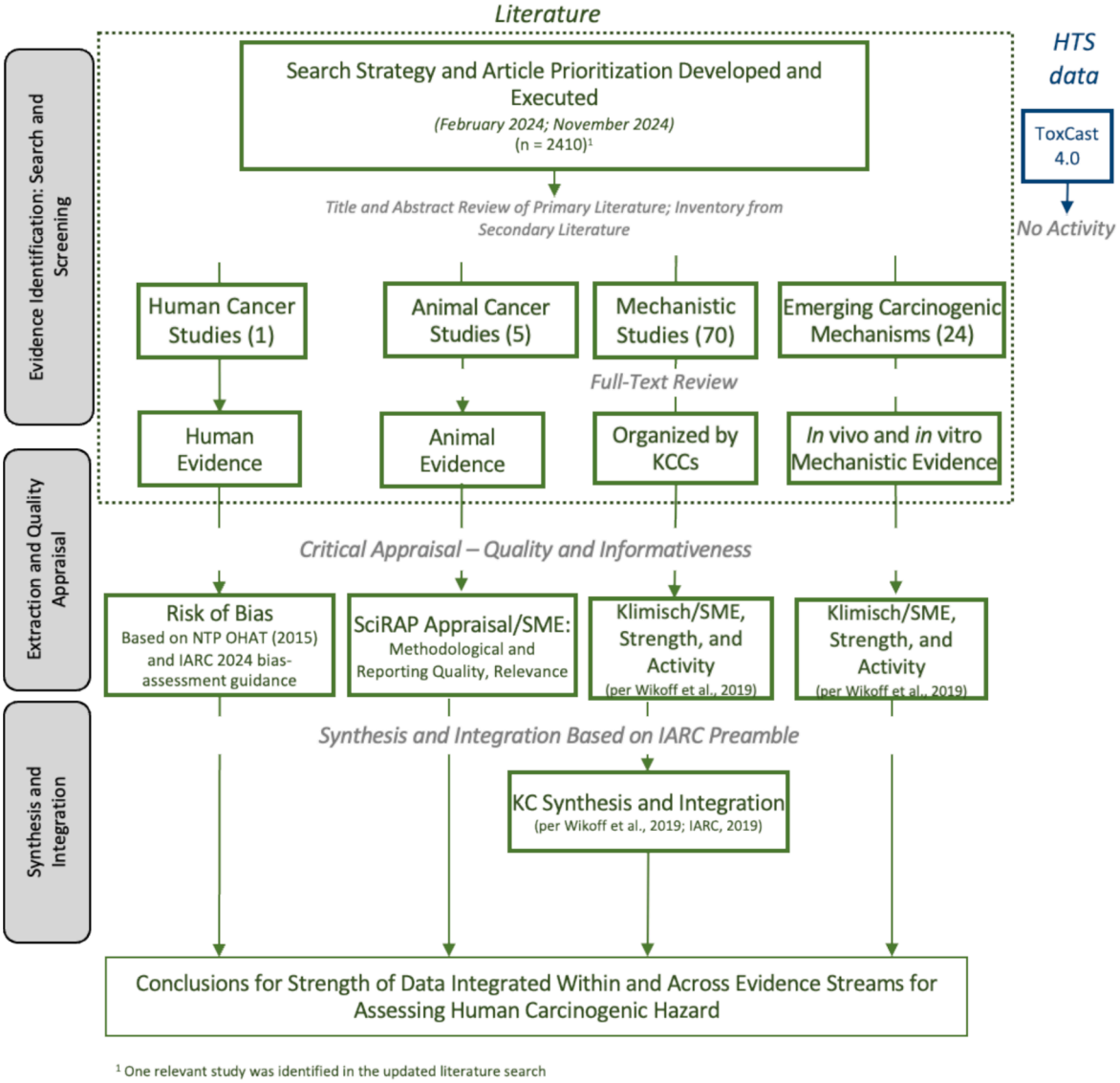

Fig. 1. Approach for systematic assessment of carcinogenic potential of MTBE. A simple representation of the volume and characterization of evidence for each stream is provided, along with additional details on the workflow described in the methods and results.

according to the KCCs, has not yet been undertaken. With the organization of mechanistic data across KCCs, this evaluation will allow for heterogenous endpoints to be considered in the evaluation of the strength of the evidence for MTBE's carcinogenic potential in humans. This systematic assessment will be the first to integrate data from human and animal cancer studies, and KCC-organized mechanistic evidence for MTBE, employing systematic review methods and critical appraisal to provide weight-of-evidence conclusions based on the comprehensive data available.

Methods

Overall approach

The approach for assessing the potential carcinogenic hazard of MTBE is outlined in Fig. 1. This systematic evaluation is built on general principles and procedures described by the IARC Preamble (2019) for conducting cancer hazard evaluations. All available evidence was evaluated, including epidemiological, experimental animal, and mechanistic data such as absorption, distribution, metabolism and excretion (ADME) data, mechanistic data for KCCs (Smith et al., 2016), and information on any emerging carcinogen mechanisms (IARC Preamble, 2019). Methods used for assessing and integrating mechanistic data were based on biological pathway-based concepts described by Smith et al. (2016) and Wikoff et al. (2019). Outcomes, such as tumor formation, observed in animal and human studies were considered in the assessment as described by the WHO IPCS (Meek et al., 2014; Meek and Wikoff, 2023).

Evidence identification – Systematic literature search and screening

A systematic review protocol for literature searching and screening is available in Appendix A. Supplementary data. The search strategy was developed with the objective of maximizing sensitivity and identifying all potentially relevant citations. Using a broad syntax, consisting of the chemical name, major synonyms, and CAS number, peer-reviewed literature searches were conducted in two citation databases, PubMed and Embase, on February 14, 2024. The literature search syntax used is provided in Appendix A. Supplementary data. Citations were deduplicated using a reference manager (EndNote) and imported to Sciome Workbench for Interactive Computer-Facilitated Text-mining (SWIFT) for prioritization by evidence stream (human, human-relevant animal models, *in vitro* or quantitative epidemiological) and health effects related to carcinogenesis. Citations meeting SWIFT prioritization criteria (i.e., evidence streams and any health outcome or any KCC) were prioritized for Title and Abstract (TiAb) review. An updated literature search was conducted in November 2024 and January 2025 with date filters from February 15, 2024 – Present (November 8, 2024) and November 9, 2024 – Present (January 21, 2025) using the same search syntax and deduplication process (Supplemental Data File A2). Titles and abstracts of identified articles from the updated literature search were manually reviewed for relevancy.

Following prioritization in SWIFT, identified TiAbs were evaluated for relevance in DistillerSR by reviewers based on categorization of the reported test article(s), evidence stream(s) (i.e., ADME, Human Cancer, Animal Cancer, KCC, Other), and which KCC (if applicable). Articles meeting criteria at TiAb, except for ADME, were advanced to full-text review and their relevance confirmed before extracting. TiAb and full-text review were conducted by a single reviewer per article. If foreign language papers were identified as relevant, these papers were translated to English for full-text review. Relevant full texts were then extracted by reviewers to capture study design parameters and outcome assessment results. Quality control KCC article extraction was performed on at least 20 % of the articles reviewed. The most recent ADME review articles, along with regulatory assessments were relied on to identify the full-text articles for summarizing metabolism similarities and differences between humans and animal models.

Additional handsearching was performed via Google Scholar and general internet searching to identify secondary literature (e.g., authoritative documents; literature reviews). These were reviewed for relevant data (e.g., citations of publicly available study reports) and used to cross-check the primary literature from the citation database searches (IARC, 1999; ATSDR 2023).

Within the protocol, (Appendix A. Supplemenary data. Supplemental Data File A1) inclusion and exclusion criteria were established based on Population, Exposure, Comparator, and Outcome (PECO) statements used to identify relevant articles (i.e., human, animal and mechanistic data for assessing MTBE's carcinogenic potential). TiAbs identified from the literature search were screened and marked for inclusion or exclusion based on the following criteria:

- Include:
  - Human epidemiological studies investigating cancer outcomes or relevant biomarkers and MTBE exposure
  - Animal cancer studies and MTBE exposure
  - Studies that evaluated the relationship between exposure to MTBE and endpoints relevant to one or more KCCs
  - Studies that evaluated the relationship between exposure to MTBE and emerging carcinogen mechanisms
  - Studies and review articles related to adsorption, metabolism, distribution or excretion (ADME) of MTBE
- Exclude:
  - Studies not obtainable, or studies that did not report original data (e.g., reviews of cancer or mechanistic data and/or commentaries)
  - Studies in foreign languages that could not be translated
  - Clinical human studies conducted for the dissolution of gallstones, unless there were measures of MTBE in blood and endpoints measured that reflected one of the KCCs.

The toxicological databases, EPA CompTox Chemicals Dashboard, and IARC KC-Hits software were reviewed for high-throughput (HT) assay data (Williams et al., 2017; Reisfeld et al., 2022). This review included ToxCast QSAR models for predicting Estrogen Receptor (ER) and Androgen Receptor (AR) binding and activity using the CERAPP (Mansouri et al., 2016) and COMPARA (Mansouri et al., 2020) consensus models, respectively as reported within the EPA CompTox Chemicals Dashboard for MTBE (EPA, 2024b). Organization for Economic Cooperation and Development (OECD) ToolBox (Version 4.7) was also used to investigate ER and AR binding and activity based on the concern that these activities might not be measurable *in vitro* due to the volatility of MTBE.

Extraction and appraisal for quality and informativeness

Data from human, animal, and mechanistic studies/assays were extracted from studies confirmed as relevant following full text review (Fig. 1). Relevant studies considered for the review were appraised for quality and reliability utilizing appropriate tools, as described below.

Cancer studies in humans

Human epidemiology studies that evaluate the association between MTBE exposure and cancer outcomes were evaluated for quality and risk of bias (RoB) using considerations described by NTP Office of Health Assessment and Translation (OHAT) (2015) and the IARC (2024) bias-assessment guidance. Epidemiology or controlled human MTBE exposure studies that provided mechanistic data were evaluated as described in Section—Mechanistic Evidence.

Cancer studies in experimental animals

Standard two-year bioassays were evaluated for cancer outcomes in experimental animals. Non-standard studies, such as an initiation-promotion study and a study in chronically exposed animals (~2-

years) that were allowed to live until their natural death, were evaluated, and used as supporting information only if determined to be reliable. Where appropriate, calculated dose levels cited by McGregor, 2006 were used for comparison across studies to evaluate dose response effects and equivalency across different studies. Study design details (e.g., species, strain, sex, duration, age, number of animals per exposure group, etc.), chemical administration and characterization (i.e., route, purity, vehicle), and results (i.e., tumor incidence, statistical significance and degree of mortality) were tabulated. Conclusions of cancer outcomes from authoritative bodies and other existing reviews were considered to provide contextual information in the final assessment of these studies (IARC, 1999; Health Canada 2006, ATSDR 2023).

All identified animal cancer studies were appraised for study quality and reliability both objectively and consistently according to general systematic review principles. Publications in which a re-evaluation of existing tissues from an original study was conducted were considered together with the original study as one study when reviewing the reporting and methodological quality. A critical appraisal tool (CAT), SciRAP (Beronius et al., 2018), was used in the appraisal, and its output interpreted for reliability categorization by one Subject Matter Expert (SME) (author IAL). Using the CAT facilitated a transparent and granular appraisal of study methods (i.e., appropriateness of the study design, study conduct) and reporting (i.e., completeness in the reporting of study design, conduct and results), guided by 30 reporting quality criteria and 18 methodological quality criteria. For each of the methodological criteria, reviewers assigned fulfillment categories based on those identified by SciRAP:

- Fulfilled: Direct and/or specific information was considered that could be understood in the context of the study. Study design and methods aligned with OECD recommendations and conduct or similar level of validity in the case where there is not a guideline equivalent.
- Partially Fulfilled: Study reported partial information on criteria to assess methodological component, or did not fully align with guideline methods.
- Not Fulfilled: Critical aspects of the methodology were not conducted.
- Not Reported: Information reported was insufficient to assess the methodological criteria.

Reliability categorization was qualitatively derived based on method conduct consistent with the criteria reported in the IARC preamble (2019), such as test substance characterization, dose monitoring, dose levels, duration and frequency, appropriate animal species/strains, number of animals per dose group, randomization, and adequate histopathological review. Consistent with approaches taken by authoritative bodies (Lahr et al., 2023), the SME (authors IAL) review was integral to assessing study informativeness and took into consideration factors not explicitly specified in the CAT, including evaluating animal health status and considering the historical control data and human relevance. Studies were categorized based on the overall assessment using SciRAP and SME. Reliability categories included:

- Reliable: Study was conducted using a method generally in alignment with standardized test guidelines e.g., OECD TG 451 (Carcinogenicity studies).
- Partially Reliable: Study was conducted using a well-defined and widely accepted method without significant deviation but did not follow standardized test guidelines. Design or reporting of study resulted in some limitations for data interpretation e.g., use of single dose levels precluding assessment of a dose–response relationship for any observed effects.
- Not Reliable: Study was not conducted using a well-defined and widely accepted method and did not follow standardized test guidelines. Design or reporting of study resulted in significant limitations for data interpretation e.g., unknown pathogen status for animals used in the study.

Mechanistic evidence

The mechanistic evidence identified and reviewed within this cancer assessment includes a summary review of ADME data collected in humans and animal models, and extraction of mechanistic data organized within the ten KCCs (Smith et al., 2016). Evidence for each KCC was evaluated for relevance, model strength, activity, and study/endpoint reliability. Additional mechanistic data identified as potential emerging issues in carcinogenesis, referred to as KCC11, were also extracted to assess their possible contribution to MTBE's carcinogenic potential.

Absorption, distribution, metabolism, and excretion (ADME). Studies were identified that provided information on the ADME of MTBE in both humans and animal models. For this assessment, focus was on the most relevant review articles, recent peer-reviewed publications not covered in previous reviews, and summaries in regulatory assessments. These sources were used to summarize MTBE's metabolism and toxicokinetic properties across different exposure routes, with emphasis on the similarities and/or differences between humans and animal models. Additionally, key MTBE metabolites were identified to consider their potential carcinogenesis role.

Mechanistic data organized by KCCs. To evaluate the mechanistic evidence, endpoint data was organized within KCCs following both the IARC Preamble (2019) and the framework described by Wikoff et al. (2019), with guidance on mapping endpoints to each KCC based on several publications (Guyton et al., 2018; Smith et al., 2020; Madia et al., 2021). Mechanistic data extraction involved systematically evaluating study details and categorizing the model (e.g., *in vitro*: human; *in vivo*: mammalian, etc.), as well as assessing model strength, activity, and study quality using Klimisch et al. (1997) criteria for *in vivo* experimental animals or *in vitro* mammalian and human primary cells or cell lines. For epidemiological studies with mechanistic data, study quality was conducted using Money et al., (2013) criteria, which directly mirrors criteria and scoring established by Klimisch with adaptations to account for elements specific to epidemiological study design considerations (e.g. exposure characterization, statistical analysis and selection, confounding, or reporting bias) (Money et al., 2013).

Extracted study details included model, rodent strain, sex, age at start of exposure, exposure route, MTBE dose level or concentration, duration of exposure, sample analyzed (i.e., tissue), and evaluations of cytotoxicity *in vitro* or systemic toxicity *in vivo*. KCC endpoint details included the KCC category (e.g., genotoxicity; oxidative stress, etc.), specific endpoint measured (e.g., chromosomal aberrations), activity status (active:1 vs. inactive: 0), and endpoint finding (e.g., no significant increase in micronucleus formation). Activity status was reported for specific doses/concentrations determined to be statistically significant from control, or a dose–response, as stated by the study authors, excluding effects occurring at cytotoxic doses *in vitro*, which were considered not reliable data for inclusion in the overall evaluation. For *in vitro* MTBE studies, control for MTBE volatility was noted for consideration of consistency and/or confidence in data. Study quality for all KCC data was evaluated according to the Klimisch or Money criteria, with reliability scores from 1 to 3: 1 = reliable without restriction; 2 = reliable with restriction; and 3 = not reliable. Guidance for determining Klimisch reliability scores was informed by the ToxRTool red criteria (Schneider et al., 2009). Critical review of oxidative stress endpoints was sorted and evaluated for reliability for measuring oxidative damage and reactive oxygen species (ROS) according to Halliwell and Whiteman (2004) and Murphy et al., (2022). Given the importance of genotoxicity data in cancer evaluation, these data were subjected to SME review focused on alignment with OECD standards widely accepted for

genotoxicity. These guidelines were also used to guide the assessment of the assays' overall reliability.

Model strength/relevancy, a proxy measure for study informativeness, was assigned based on a weighting structure adapted from Wikoff et al. (2019), with modifications for Ames assays (non-mammalian) because of their strong ability to predict mutagenicity. In this assessment, data from exposed humans were rated highest with a score of 8. This was followed by *in vitro* human primary cells and *in vivo* mammalian study data with a score of 4. *In vitro* human cell lines, non-human mammalian primary cells and cell lines, and Ames assays received a score of 2, while all other *in vitro* and *in vivo* non-mammalian models scored 1. This systematic mapping enabled a quantitative integration of mechanistic activity (if/where observed) for each KCC and guided the evaluation of evidence strength within each KCC by considering critical study aspects (i.e., repeat dose or multiple endpoint measurements—versus single dose or single time point; suitability of dosing range; extent of concurrent toxicity; limitations of the test system; and characterization of test article).

Data for obesity, diabetes, and related endpoints reported in human studies, animal *in vivo* studies, and any relevant *in vitro* studies followed the same extraction and study quality evaluation approach as described for the other KCCs. Any of these epidemiology studies in which mechanistic endpoints were reported were all included. Pertinent endpoints included liver weight, body mass index (BMI), serum glucose, and blood triglycerides. Data from all evidence streams were evaluated collectively for consistent associations between MTBE exposure and changes linked to obesity and diabetes, including changes to insulin sensitivity, insulin resistance, glucose metabolism, BMI, lipid metabolism, and gastrointestinal microbiome, among others.

Consideration of strength of mechanistic activity within each model type per KCC. Within each KCC, mechanistic data extracted was reviewed for consistency across study types and endpoints. Studies considered reliable (Klimisch or Money score of 1 or 2) were included in data integration. Although mechanistic data from studies considered not reliable (Klimisch or Money score of 3) may be discussed within the evaluation of each KCC, for transparency and to highlight why the data would not be used in the consideration of strength of activity. All studies reviewed and extracted, along with reliability justification are provided in the Appendix A. Supplementary data. (Tables S1-S13). Data were evaluated for strength of activity first within model types, such as exposed humans, *in vitro* human primary cells and tissues, *in vivo* animal studies, *in vitro* human cell lines or non-human mammalian primary cells or cell lines. The *in vitro* non-mammalian Ames assays was categorized with *in vitro* non-human mammalian cells based on its predictive ability for mutagenic response. Strength of activity was evaluated (per model type) across endpoints in reliable studies as consistent activity, inconsistent activity, minimal/weak activity, overall negative activity, or no data or data in unreliable studies.

Integration of mechanistic activity across each KCC. Strength of the mechanistic data was classified with consideration of the IARC framework (IARC, 2019), with some modifications:

1. Strong evidence: Assigned when the overall evidence base (across each KCC) was coherent with consistent activity in reliable studies/endpoints. This included: a) strong evidence in exposed humans; b) strong evidence in human primary cells or tissues (human receptors and enzymes, etc.); or c) strong evidence in experimental *in vivo* animal models supported by evidence in several studies in human primary cells or tissues.
2. Limited evidence: Assigned when the overall evidence base (across each KCC) was suggestive, with studies covering a narrow range of experiments, endpoints, and/species. Also assigned because of inconsistencies in similar studies, and /or incoherence across studies of different endpoints or different experimental systems.
3. Inadequate evidence: Assigned when there were few or no data available, or when there were unresolved questions about the adequacy of the study design, conduct, or interpretation, or the results were negative.

Overall, strength of mechanistic data. The overall strength of the mechanistic activity was evaluated by examining the robustness of the data within each KCC, as well as identifying individual KCCs considered to have strong findings, or the combination of two of more KCCs that together supported a strong indication of carcinogenic activity associated with MTBE exposure. MTBE metabolism in humans and animals was also considered in reliability of model type where activity occurred for overall strength of data. Classification of overall strength of the mechanistic data was considered Strong, Limited, or Inadequate, similar to the description IARC's Preamble (IARC Preamble, 2019).

Integration across evidence base

An overall assessment of MTBE's potential for a carcinogenic outcome in humans was conducted by integrating the totality of the data across evidence streams (i.e., human cancer studies, animal cancer studies, and mechanistic data). A systematic approach, grounded in weight-of-evidence considerations and established scientific principles, was used to evaluate the carcinogenic potential within and across these evidence streams. Tumors from standard, reliable cancer bioassays were considered in this evaluation, with data from non-standard studies included as additional support, when appropriate. For the mechanistic data, the strength across each KCC and/or integrated together across KCCs was evaluated to assess the biological plausibility of a carcinogenic response in humans. Similarities in MTBE metabolism in humans and animal models were considered in this assessment. Specific tumors identified in animal studies, in which the mechanism or mode of action, is shown not to, or unlikely to operate in humans (IARC, 1999), are not considered in the overall evaluation. The assessment triangulated study strengths and weaknesses, with concepts of consistency, dose–response, magnitude of response, and relevance and reliability across the evidence base considered as needed to evaluate the overall carcinogenic hazard posed by MTBE exposure to humans.

Results

A total of 2409 citations prioritized via SWIFT were screened for relevancy via TiAb from the initial literature search in February 2024. An additional 14 papers were identified from hand-searching giving a total of 2423 publications. Of these, 136 publications were retrieved and reviewed for relevance at the full-text level. The updated literature searches conducted in November 2024 and January 2025 resulted in identification of 2 recently published studies. No relevant human cancer studies were identified in the initial search, however, one relevant human cancer study was identified in the updated literature search conducted in November 2024 along with one mechanistic study in the literature search conducted on January 21, 2025. Overall, five studies in experimental animal models evaluated cancer outcomes associated with MTBE exposure. Seventy-three articles contained mechanistic data, and 25 articles reported emerging carcinogenic mechanisms related to obesity, type II diabetes, and/or insulin resistance (Fig. 1).

Cancer studies in humans

One human epidemiological study that assessed cancer associated with environmental MTBE along with other chemicals was identified in the updated literature search conducted in November 2024 (Rodriguez et al., 2024). No studies of occupational exposure settings were

Table 1
Incidence of significant tumors reported in animal cancer studies.

Reference	Study Design Species, Strain (sex) Age at Start Duration	Route Purity Vehicle Dose(s) No. of Animals at Start % of Animals Surviving in Each Dose Group	Tumor Incidence[a] (# tumors/# examined)
Bird et al., 1997	Standard chronic bioassay	Inhalation	No significant tumor responses attributed to MTBE exposure in male mice. Full necropsy and histopathological examination performed, including brain, liver, kidney and testis.
Burleigh-Flayer et al., 1992	Mouse, CD-1 (M&F)	Purity: >99 %	Hepatocellular adenoma
(Study report)	Age: 49–56 days	Air	M: 11/49, 11/50, 9/50, 12/49
See Fig. 2 and S1; for SciRAP Evaluation	Duration: 6 h/day, 5 days/week for 18 months	0, 400, **3000, 8000 ppm**[b]	F: 2/50, 1/50, 2/50, **10/50**[c]
		Calculated dose: M: 0, 210, 1550, 4150 mg/kg bw, 5 days/week F: 0, 250, 1850, 4950 mg/kg bw, 5 days/week 50 M/ 50F for each dose level M: 67 %, 78 %, 65 %, 51 % F: 73 %, 82 %, 77 %, 67 %	Hepatocellular carcinoma M: 2/49, 4/50, 3/50, 8/49 F: 0/50, 1/50, 0/50/ 1/50 Combined adenoma and/or carcinoma M: 12/49, 12/50, 12/50, 16/49 F: 2/50, 2/50, 2/50, 11/50
Bird et al., 1997	Standard chronic bioassay	Inhalation	No significant tumor responses attributed to MTBE exposure in female rats. Full necropsy and histopathological examination performed including brain, liver, kidney and testis.
Chun et al.,1992 (Study Report)	Rat, F344 CDF (M&F)	Purity: >99 %	Renal adenoma
See Fig. 2 and S1; for SciRAP Evaluation	Age: 52 days	Air	M: 1/50, 0/50, 5/50, 3/50
	Duration: 6 h/day, 5 days/week for 104 weeks	0, 400, **3000, 8000 ppm**[b]	Renal carcinoma
		Calculated dose: M: 0, 40, 310, 830 mg/kg bw, 5 days/ week F: 0, 60, 450, 1190 mg/kg bw, 5 days/week 50 M/ 50F for each dose level M: 26 %, 12 %, 12 %, 18 % F: 60 %, 54 %, 46 %, 50 % Study terminated at week 82 (3000 ppm) and week 97 (8000 ppm). CPN considered primary reason for early deaths.	M: 0/50, 0/50, 3/50, 0/50 Renal adenoma and/or carcinoma M: 1/50, 0/50, **8/50**[d], 3/50 Testicular interstitial (Leydig) cell adenoma M: 32/50, 35/50, **41/50**[c], **47/50**[d]
Dodd et al., 2013	Standard chronic bioassay	Drinking water	No tumor responses attributed to MTBE exposure in female rats. Full necropsy and histopathological examination performed, including brain, liver, kidney, and testis.
See Fig. 2 and S1; for SciRAP Evaluation	Rat, Wistar Han GALAS (M&F)	Purity: >99 %	Brain astrocytoma
	Age: 6–8 weeks Duration: 24 months	Reverse osmosis purified water 0, 0.5, 3, 7.5 (M), 15 (F) mg/mL Calculated average dose: M: 0, 25, 140, 333 mg/kg-bw/day F: 0, 49, 232, 1,042 mg/kg-bw/day 50 M/ 50F for each dose level % survival not provided; no significant difference in survival between exposed and control rats (M&F)	M: 1/50, 1/50, 1/50, **4/50**[e] F: 0/50, 0/50, 0/50, 1/50
Moser et al., 1996	Non-standard assay. Initiation-promotion	Inhalation	No tumor responses in liver attributed to MTBE exposure in female mice following 16- or 32-weeks exposure.
	Mouse, B6C3F1 (F)	Purity: >99 %	
See Fig. 2 and S1; for SciRAP Evaluation		Air	32 weeks:
	Age: 8 weeks		Hepatocellular adenoma
	Duration: 6 h/day, 5 days/week for 16 or 32 weeks	0, 8000 ppm with a single 5 mg/kg-bw i.p. injection of DEN	F[f]: 88 % [10/12], 90 % [11/12]
		10–12F for each dose level	Hepatocellular carcinoma F[f]:50 % [6/12], 40 % [5/12]
		F: 96 %, 88 %	

(*continued on next page*)

Table 1 (*continued*)

Reference	Study Design Species, Strain (sex) Age at Start Duration	Route Purity Vehicle Dose(s) No. of Animals at Start % of Animals Surviving in Each Dose Group	Tumor Incidence[a] (# tumors/# examined)
Belpoggi et al., 1995;1997; 1998	Non-standard chronic bioassay	Oral Gavage	Full necropsy and histopathological examination performed including brain, liver, kidney and testis.
See Fig. 2 and S1; for SciRAP	Rat, Sprague-Dawley (M&F)	Purity: >99 %	Leydig cell testicular tumors[f,g]
Evaluation; Not Reliable	Age: 8 weeks Duration: 4 days/week for 104 weeks with animals observed until natural death	Olive oil 0, 250, 1,000 mg/kg-bw/day, 4 days/week 60 M/ 60F for each dose level [M: 30 %, 30 %, 55 % at 104 weeks of age] [F: 50 %, 40 %, 30 % at 104 weeks of age]	M: 2/26, 2/25, **11/32** [g] Lymphoma and leukemia[h] M: 10/59, 9/59, 7/58 F: 2/58, **6/51**[i], **12/47**[j]

Notes on following page.

[a] Tumors with increased statistical significance shown in bold.

[b] Exposure concentration (3000 and 8000 ppm) exceeded maximum tolerated dose (MTD) (based on survival. mortality, body-weight decreases, and CNS depression).

[c] $p < 0.01$ Fisher's exact test,

[d] $p < 0.05$ Fisher's exact test; **Note: Incidence of testicular tumors are within historical control range, although statistically different from concurrent control (on the low end).**

[e] $P = 0.032$ Cochran–Armitage trend test (one-sided); $P = 0.037$ poly 3 adjusted trend (one-sided); $P < 0.181$ Fisher's exact test (one-sided) (0 versus 7.5 mg/mL).

[f] Values in square brackets indicate tumor incidence estimated from data in paper.

[g] Number of rats examined adjusted to be the number alive at the age of 96 weeks (Leydig cell) or 32 weeks (leukemia and lymphoma) when the first neoplasms were observed.

[h] $p < 0.05$ Hoel and Walburg.

[i] $p < 0.1$ Mantel and Cox.

[j] $p < 0.01$ Mantel and Cox.

identified. Rodriguez et al. (2024) reported on a pilot study in which concentrations of 173 environmental contaminants, including MTBE, were estimated based on measurements of 18 non-specific metabolites in urine of 20 pancreatic ductal adenocarcinoma (PDAC) patients and compared to 20 healthy controls. MTBE exposures were not measured directly in the urine and were estimated based on one of the 18 metabolites identified (2-hydroxyisobutyric acid), a metabolite of MTBE via TBA. Methods for quantitatively estimating MTBE exposures, especially in isolation from other contaminants of interest, from this single metabolite were not described. The authors did report a significant mean increase in MTBE concentration between cases and controls. However, interpreting these findings for hazard characterization is limited, as the single urine samples collected at baseline are unlikely to be representative of long-term exposures to MTBE; absorbed MTBE and its key metabolite, *tert*-butyl alcohol (TBA), have clearance half-lives of < 1 day in humans (Amberg et al., 2001; Nihlen et al., 1998). Therefore, the measured concentrations of the MTBE-related metabolite (2-hydroxyisobutyric acid) in urine are unlikely to be representative of long-term exposures, nor are they likely to be etiologically relevant to the observed PDAC incidence, which has a latency period of > 10 years. (See Appendix A. Supplementary data. Table S1), for additional information regarding critical appraisal). Therefore, since reporting on this study was considered not reliable based on clarity in the relationship between exposure to MTBE and tumor outcome, overall, there was "inadequate" data for consideration of cancer in humans. Epidemiology studies identified as informative for providing mechanistic data, including the evaluation of "emerging" cancer outcomes pathways, were reviewed within the mechanistic data section.

Cancer studies in experimental animals

Five MTBE cancer studies were identified from which data (significant tumor types) were reviewed and summarized (Table 1**)**. Three standard 2-year studies were identified, two conducted in rats (Chun et al., 1992; Bird et al., 1997; Dodd et al., 2013) and one study in mice (Burleigh-Flayer et al., 1992; Bird et al., 1997), along with two non-standard studies: (1) an initiation-promotion study in mice (Moser et al., 1996a), and (2), a cancer study in rats in which MTBE was administered orally for two years and observed until the animal's natural death (Belpoggi et al., 1995;1997; 1998).

The SciRAP assessment was summarized for an overall categorization of reliability, in which the contributions of reporting and methodology attributes were considered in this cancer hazard assessment. Fig. 2 provides the SciRAP assessment of study design (methodology) as well as an overall summary of each study's strengths and limitations as determined by SME assessment. The SciRAP assessment of the study reporting criteria is provided in the Appendix A. Supplementary data Figure S1. Of the five animal studies evaluated, all three standard cancer bioassays were considered reliable. The initiation-promotion study (non-standard) was considered partially reliable (Moser et la., 1996a), and the remaining non-standard cancer study was considered not reliable (Belpoggi et al., 1995;1997; 1998). In the latter study, rats were bred in-house, in a facility not verified as pathogen free. The potential presence of *Mycoplasma pulmonis*, known to cause chronic inflammatory reactions, may have played a role in the observed tumor responses (Schoeb et al., 2009; Gift et al., 2013; Elmore et al., 2023), which was a significant limitation of this study. These shortcomings make using this study difficult to assess MTBE's potential carcinogenic activity, an opinion supported by other evaluations (IARC, 1999; Health Canada, 2006; IPCS, 1998). Given the lack reliability, the data for Belpoggi et al. study are reported in Table 1 but not discussed below.

In the reliable inhalation study in which CD-1 mice were exposed to 0, 400, 3,000 and 8,000 ppm MTBE (calculated dose: 0, 210, 1550, 4150 mg/kg-body weight (bw) (male); 0, 250, 1850, 4950 mg/kg-bw (female), 6 hr/day, 5 days/week) for 18 months, a significant increase in hepatocellular adenoma incidence was reported in female mice (2/

	Chun et al., 1997; Bird et al., 1997 (Rat)	Burleigh-Flayer et al., 1992; Bird et al., 1997 (Mouse)	Dodd et al., 2012 (Rat)	Belpoggi et al., 1995; 1997; 1998 (Rat)	Moser et al., 1996 (Mouse)
Test compound and controls					
MTBE was unlikely to contain any impurities that may affect toxicity	F	F	F	F	F
The selected vehicle did not interfere with the absorption, distribution, metabolism, excretion, or toxicity of MTBE.	F	F	F	F	F
A concurrent negative control group was included.	F	F	F	F	F
Animal model and housing conditions					
The selected animal model was appropriate for the investigation of carcinogenesis.	F	F	F	NF	F
The housing conditions were appropriate for an investigation of carcinogenesis in the selected animal model.	F	F	F	ND	F
The number of animals per sex in each cage were appropriate for an investigation of carcinogenesis.	PF	PF	F	NF	PF
The test system (e.g., cage) was unlikely to contain contaminants that could affect study results.	F	F	F	ND	PF
Dosing and administration of the test compound					
Animals were assigned randomly to different treatments.	F	F	F	PF	F
The route of administration was appropriate to investigation of MTBE carcinogenic potential.	F	F	F	PF	F
MTBE was administered for a duration appropriate to the investigation of carcinogenesis.	F	F	PF	NF	F
MTBE was administered with a frequency appropriate to the investigation of carcinogenesis.	PF	PF	F	NF	PF
Data collection and analysis					
Animals were allocated randomly to different tests and measurements.	F	F	F	F	F
Tests methods used could generate reproducible results.	F	F	F	F	F
Data were collected at timepoints appropriate to the investigation of carcinogenicity.	PF	PF	F	NF	F
Measurements were conducted in a sufficient number of animals per dose group.	F	F	F	PF	PF
Statistical methods used in the analysis were appropriate to the study design.	PF	PF	F	NF	PF

Fig. 2. Animal Cancer Studies; SciRAP methodology reporting; Reliability based on granular appraisal of methodology attributes using SciRAP with overall reliability using SME to interrogate SciRAP evaluation with identified strengths and study limitations. The SciRAP reporting heatmap is provided in Appendix A. Supplementary data. Figure S2.

50 in control mice versus 10/50 in the 8,000 ppm group), with no increase in hepatocellular adenoma incidence in male mice or in hepatocellular carcinomas in either male or female mice (Table 1) (Burleigh-Flayer et al., 1992; Bird et al.,1997). Based on findings of high mortality (males only), and reduced body and organ weights, 8,000 ppm MTBE was considered to have exceeded the maximum tolerated dose (MTD) in male and female mice. In a follow up (partially reliable) study to investigate if MTBE would promote hepatocellular adenomas in exposed female mice, an initiation-promotion study was conducted using B6C3F1 female mice initiated with a single intraperitoneal injection of the liver tumor initiator N-nitrosodiethylamine (DEN) (5 mg DEN/kg), followed by 32 weeks of exposure to 8,000 ppm MTBE (6 hr/day, 5 days/week) (Moser et al., 1996a). This study reported little difference in hepatocellular adenoma incidence in DEN/control (88%) versus DEN/MTBE (90%) mice (Table 1), and did not provide insight into the ability of MTBE to produce liver adenomas in chronically exposed CD-1 female mice (Burleigh-Flayer et al., 1992; Bird et al., 1997), and questioning the relevance of this tumor endpoint present in only female mice exposed to extremely high concentrations of MTBE (8000 ppm).

In an inhalation study conducted in F344 [CDF] rats determined to be reliable, an increase (not dose-related) of renal adenoma/carcinoma incidence was observed in the mid- and high-dose males, but not in female rats exposed to 0, 400, 3,000 and 8,000 ppm MTBE for 104 weeks (calculated dose: 40, 310, 830 mg/kg-bw (males) or 60, 450, 1190 mg/kg-bw (females), 6 hr/day, 5 days/week) (Chun et al., 1992; Bird et al., 1997). The highest tumor incidence occurred in males exposed to 3,000 ppm MTBE (8/50), compared to 8,000 ppm (3/50) MTBE and controls (1/50) (Table 1). The male rats in this study exhibited significant mortality, as well as decreased body and organ weight, suggesting the MTD had been exceeded. At the mid- and high-dose levels, high mortality resulted in early termination of these groups (weeks 82 or 97 respectively) and may have contributed to lower tumor incidence than if the animals had survived to the end of the study. Early male deaths were attributed to chronic progressive nephropathy (CPN). In another cancer bioassay conducted in rats (Wistar Han; GALAS) and also considered reliable, male and female rats were exposed to MTBE via drinking water at concentrations of 0, 0.5, 3, 7.5 (male), 15 (female) mg/mL (calculated dose 0, 25, 140, 333 mg/kg-bw/day in males and 0, 49, 232, 1,042 mg/kg-bw/day in females). No neoplastic lesions were observed in kidneys of males or females following 24 months of MTBE exposure (Dodd et al., 2013). The only tumor endpoint identified in this MTBE study was a low, but increased astrocytoma incidence observed in the brain of male, but not female, rats exposed to 333 mg/kg-bw/day MTBE (4/50 compared to 1/50 in the control group) (Dodd et al., 2013) (Table 1). An increased incidence of brain tumors in rats is an uncommon finding and rare in untreated rodents (Rice and Wilbourn, 2000; Sills et al., 1999). It is of interest to note that brain tumors are not detected in the other chronic MTBE bioassays conducted at higher dose levels (Bird et al., 1997).

An increased interstitial (Leydig) cell adenoma incidence was observed in the reliable MTBE inhalation study conducted in F344 rats,

	Chun et al., 1997; Bird et al., 1997 (Rat)	Burleigh-Flayer et al., 1992; Bird et al., 1997 (Mouse)	Dodd et al., 2012 (Rat)	Belpoggi et al., 1995; 1997; 1998 (Rat)	Moser et al., 1996 (Mouse)
SciRAP Scores	Reporting Quality: 91.7% Method Quality: 88.2%	Reporting Quality: 91.7% Method Quality: 88.2%	Reporting Quality: 96.7% Method Quality: 97.1%	Reporting Quality: 58.3% Method Quality: 44.1%	Reporting Quality: 81.7% Method Quality: 79.4%
Overall Reliability Assessment based on SME Evaluation	**Reliable**	**Reliable**	**Reliable**	**Not Reliable**	**Partially Reliable**
Criteria used by SME to assign overall reliability category	**Strengths:** Generally, in compliance with OECD TG 451 and GLP requirements. Rats exposed to high doses of MTBE. Verification of chamber atmosphere performed during exposure and assessment test system contaminants performed. 50 rats/sex/dose group sufficient for statistical analysis **Limitations:** Significant mortality resulted in early termination of mid-and high-dose male F344 rats at 97 and 82 weeks respectively. Increased mortality attributed to high incidence of CPN (a common age-related finding in rats, especially F344).	**Strengths:** Generally, in compliance with OECD TG 451 and GLP requirements. Mice exposed to high doses of MTBE. Verification of chamber atmosphere performed during exposure and assessment test system contaminants performed. 50 mice/sex/dose group sufficient for statistical analysis **Limitations:** Increased mortality in high dose male CD-1 mice attributed to uropathy but within the bounds of historical control incidence.	**Strengths:** Generally, in compliance with OECD TG 451 and GLP requirements. Rats exposed to high doses of MTBE (330 mg/kg-bw/day (M) or 1,042 mg/kg-bw/day (F). Ambient air in room monitored for off-gassing and exhalation of MTBE. MTBE/TBA contamination of drinking water routinely assessed. 50 rats/sex/dose group sufficient for statistical analysis. **Limitations:** Lack of institutional historical control data for Wistar rats (same time-frame and dose route). Pododermatitis occurred in male rats (all dose groups) and was severe enough to cause removal from study at later timepoints. Control cage MTBE air concentrations averaged <0.11 ppm.	**Strengths**: Total number of animals in exposures groups were comparable to OECD TG 451 **Limitations**: Not conducted under GLP conditions or in a standard cancer bioassay. Pathogen status of animals not provided; health monitoring surveillance not provided. Use of animals bred 'in-house' noted to have high incidence of infection with of *M. pulmonis;* influences tumor outcome. Presentation of tumor data did not follow standard practices in combining proliferative lesions. Lack of specific historical control data (same timeframe, dose route strain) provided. Statistical analyses failed to account for differences in survival age.	**Strengths**: Model with a predictable liver tumor development; allowed experimental testing MTBE as a liver tumor promoter **Limitations:** Female mice were tested at a single exposure concentration. Study length was minimally sufficient to produce liver tumors, the incidence of hepatocellular carcinomas in control mice was low. Study design was not a standard cancer bioassay; not clear whether this models was sufficiently sensitive to detect a positive tumor response.

Fig. 2. (*continued*).

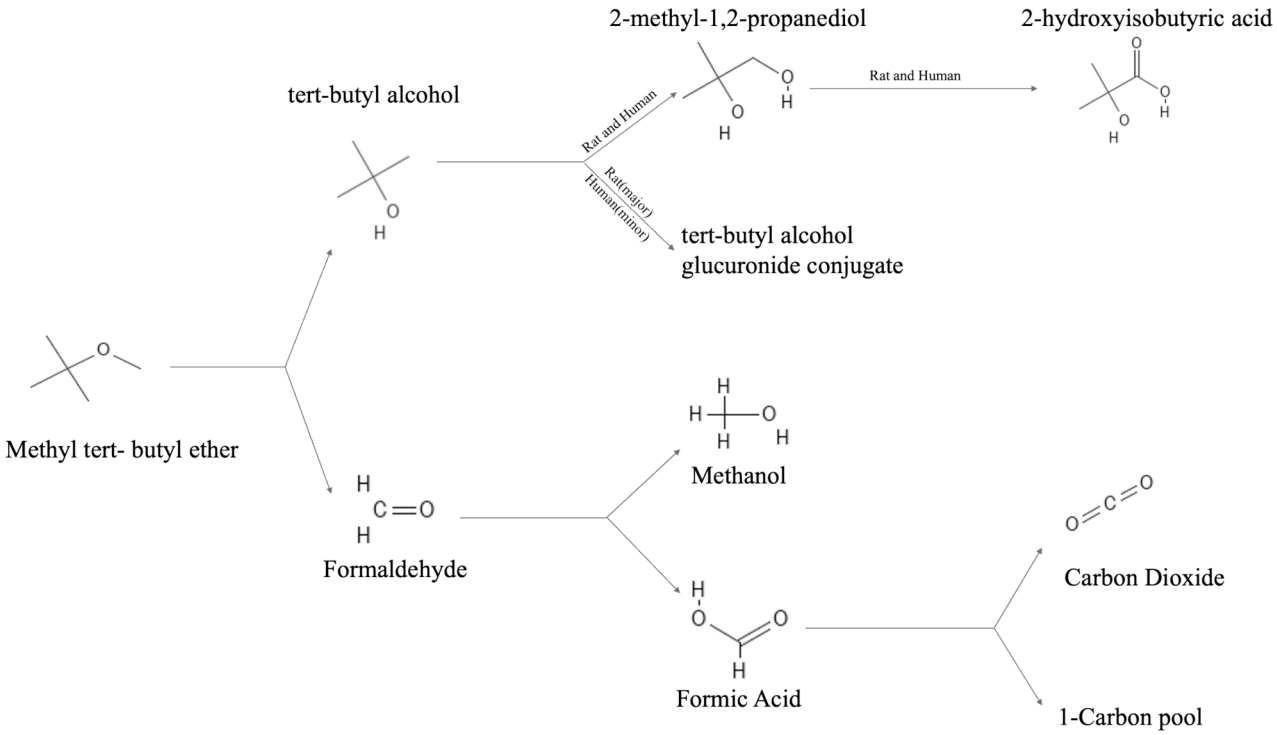

Fig. 3. Proposed metabolic pathway for MTBE in humans and rats. (Sources include Health Canada, 2006; McGregor, 2006; Bogen and Heilman, 2015; Bus et al., 2022; ATSDR, 2023).

as described above (Chun et al., 1992; Bird et al., 1997). A statistically significant increase of testicular interstitial cell adenomas was observed in rats exposed to 3,000 and 8,000 ppm MTBE (82% and 94%, respectively) compared to the control group (64%) (Table 1). The highest exposure concentration (8,000 ppm) exceeded the maximum tolerated dose (MTD), and the tumor incidence at this mid-exposure concentration (3,000 ppm) is within the historical control values for male F344 rats with the incidence in the control group within the lower historical incidence rates for this tumor type (Bird et al., 1997). The high spontaneous incidence of Leydig cell tumors in male rats means this tumor endpoint in not useful in identifying potential carcinogenic responses in the testes (Maronpot et al., 2016).

Mechanistic evidence

Metabolism and toxicokinetics of MTBE in humans and animal models

MTBE metabolism and toxicokinetics in humans and animal models has been extensively studied, with findings summarized in several reviews and regulatory assessments (Health Canada, 2006; McGregor, 2006; Bogen and Heilman, 2015; Bus et al., 2022; ATSDR, 2023) (Fig. 3). For this assessment, the similarities and differences between the metabolism and toxicokinetic parameters of MTBE in humans and rodents are summarized for support in interpreting the one human study identified, along with animal studies and the mechanistic data.

Overall, the biotransformation of MTBE is generally similar among rats and humans and between sexes (Amberg et al., 1999; Bernauer et al., 1998; McGregor, 2006; ATSDR, 2023), with MTBE being rapidly absorbed when exposed via inhalation or orally, and demethylated via cytochrome P450 enzymes to form equimolar amounts of TBA and formaldehyde (Fig. 3; ATSDR, 2023). Further, TBA metabolism results in TBA glucuronidation (in rats) and the formation of 2-methyl-1,2-propanediol, which is oxidized to 2-hydroxyisobutyric acid. These metabolites are eliminated in the urine of MTBE exposed humans and animal models (Bernauer et al., 1998; Health Canada, 2006; McGregor, 2006; Bogen and Heilman, 2015; Bus et al., 2022; ATSDR, 2023). If metabolism of formaldehyde is not saturated, methanol or formic acid may result, with formic acid being metabolized to CO_2 and potentially contributing a carbon atom to the 1-carbon pool.

While MTBE metabolism in humans and rats is similar, liver microsomes from rodents showed activity rates approximately twice as fast as those in humans (Hong et al., 1997; Health Canada, 2006). Based on the extreme differences in dose levels administered to rats and humans, metabolic saturation is only reported in rats (Miller et al., 1997; Bogen and Heilman, 2015; ATSDR, 2023) and not demonstrated in human studies (Bogen and Heilman, 2015), likely because of lower doses used. Whether human volunteers exhibit first-pass metabolism following oral exposure (Amberg et al., 2001; Prah et al., 2004; Health Canada, 2006; Bogen and Heilman, 2015; ATSDR, 2023) remains unclear, likely because of a combination of differences in dose levels and sampling times across studies and routes of exposure.

In general, rats eliminate MTBE and its metabolites faster than humans. Elimination half-lives of MTBE in rats are approximately 0.5 h, following single and repeated inhalation exposures (Miller et al., 1997; ATSDR, 2023), and $<$ 1 day in humans when considering the primary phase (Amberg et al., 1999; Nihlén et al., 1998; Prah et al., 2004; ATSDR, 2023). The plasma half-life of MTBE in rats is 0.52 and 0.79 h following oral administration of 40 and 400 mg/kg MTBE to, respectively (Miller et al., 1997; ATSDR, 2023); the primary phase for human half-lives are 0.8 h and 0.7 h following oral doses of 5 mg or 15 mg MTBE, respectively (Amberg et al., 2001; ATSDR, 2023). Volume of distribution for rats is roughly equivalent to body weight (bw) (Miller et al., 1997; McGregor, 2006) but is ~ 3.7 times bw for humans because of differences in fat volume (Nihlén et al., 1998; McGregor, 2006).

Mechanistic data organized and evaluated by KCC

Mechanistic data for MTBE were organized via the KCC framework and evaluated to assess the biological plausibility of a response in humans. This section also includes a review of a mechanism (or mode of action) of tumor formation in animal models that does not operate in humans.

Mechanistic data reviewed and extracted, including model type, activity, and reliability, is available in Appendix A. Supplementary data,

Tables S1-S13 with overall findings summarized in **Fig. S2**. Summary tables are provided below for KCC2, and KCC10 since these KCCs were critical in evaluating the overall strength of the mechanistic data. Summary tables are also presented for KCC5 for transparency since many of the studies/endpoints were eliminated for integration based on the use of methodology that was not considered reliable as documented in Table S6. Data included for integration across each KCC were assessed based on activity in assay categories (i.e., *in vivo* animal models, *in vitro* assays in primary cells or cell lines, etc) to evaluate MTBE's carcinogenic potential, with only reliable (Klimisch score of 1 or 2) studies considered. Data were evaluated at dose levels that did not produce systemic toxicity (*in vivo* studies) or cytotoxicity (*in vitro* assays), since these effects would interfere with endpoint interpretation. For *in vitro* studies, assays that controlled for volatility of MTBE in the test system provided confidence that MTBE was being tested, since MTBE would likely volatize out of the test system if not controlled, potentially leading to false-negative findings. If *in vitro* assays showed activity, but did not describe how MTBE's volatility was contained, and/or if cytotoxicity was not measured, their activity, whether positive or negative, was questioned since exposure concentration and/or cytotoxicity interference was unclear. Based on the limited information available in most of these *in vitro* assays for concentration of MTBE, however, all *in vitro* assays that reported activity are considered for integration as long as if cytotoxicity was measured. As a study category, i.e., *in vitro,* the confidence in these results was considered based on inability to confirm concentration of MTBE in the system. Although *in vivo* and *in vitro* non-human mammalian studies were reviewed (See Appendix A. Supplementary data, Tables S1-S13), only the Ames assay data, in which mutagenicity is evaluated *in vitro* (KCC2 – is genotoxic), were considered in the final assessment.

KCC1: "Is electrophilic or can be metabolically activated to an electrophile" (Appendix A. Supplementary data, Table S2)

In vitro non-human mammalian cells. In a reliable study, Casanova and Heck (1997) demonstrated that a low level of DNA-protein cross-links (DPX) or RNA-formaldehyde adducts (RFA) formed when ^{14}C-MTBE (0.33–6.75 mM) was incubated in hepatocytes isolated from CD-1 or B6C3F1 mice, or F344 rats for two hours, without concentration-dependent formation. Formaldehyde is the metabolite responsible for formation of crosslinks/adducts. When MTBE was administered to CD-1 mice to induce the metabolism of MTBE to formaldehyde, the level of crosslinks formed were not changed in hepatocytes isolated from these mice. In this *in vitro* experiment, the volatility of MTBE was controlled during incubation, providing confidence in the results. Considering the lack of concentration-dependent differences, these findings support that the metabolism of MTBE to formaldehyde is slow compared to the rate of formaldehyde metabolism. Several *in vitro* studies measured the formation of DNA adducts or crosslinks with incubation of MTBE with either double-stranded calf thymus DNA (Ghasemi and Ahmadi, 2014) or rat lung and liver cells (Yuan et al., 2005) at various concentrations, however based on either lack of information in reporting and methodological concerns with these assays they were not considered reliable for consideration in the overall findings of this KCC (Ghasemi and Ahmadi, 2014; Yang et al., 2005).

In vivo mammalian studies. In two *in vivo* mammalian studies, DNA adducts were identified following exposure to radiolabeled MTBE (Du et al., 2005; Yaun et al., 2007). Yuan et al. (2007) reported a dose dependent increase in DNA adducts in liver, lung, and kidney six hours

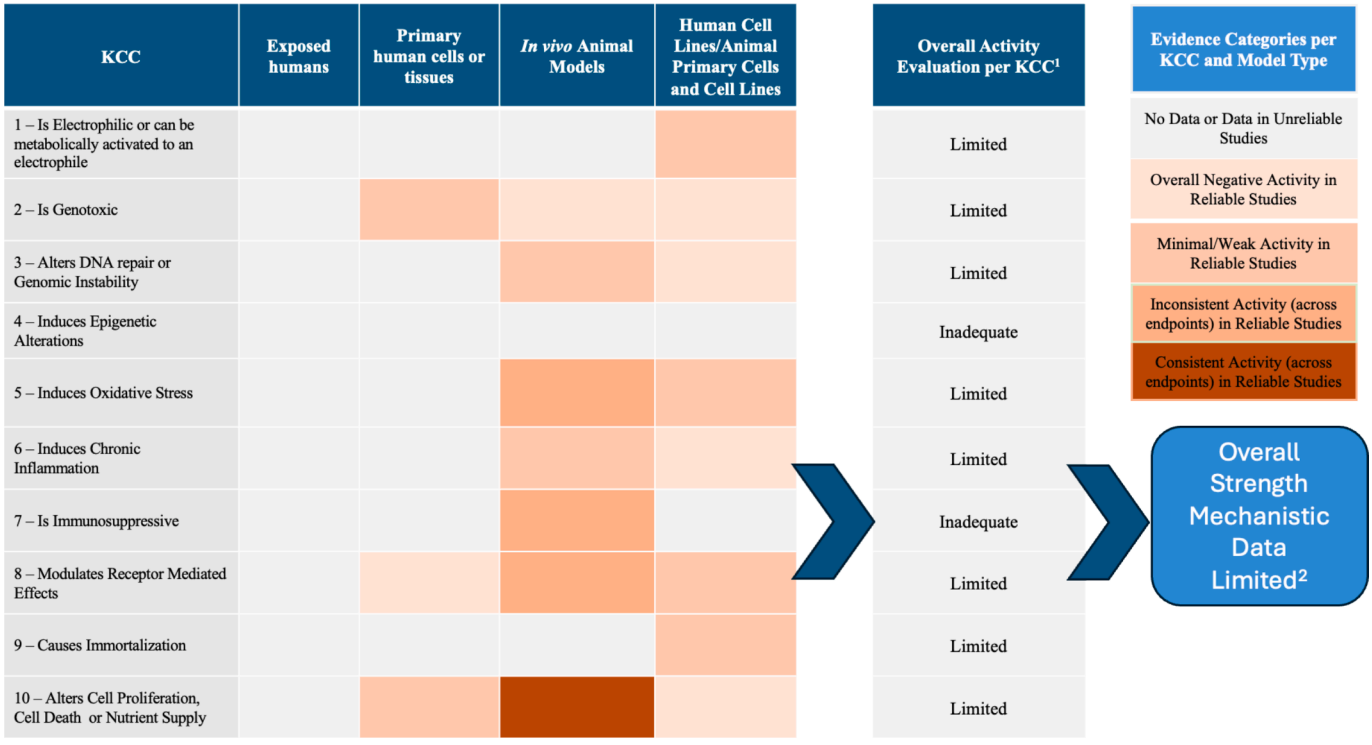

Fig. 4. Evaluation of overall mechanistic activity [1] Classification of overall strength of mechanistic data may depend on strong activity in one or more KCC's. Similar to IARC (Preamble 2019); (a) Strong evidence in exposed humans, (b) strong evidence in human primary cells or tissues or (c) strong evidence in experimental *in vivo* animal models supported by evidence in several studies in human primary cells or tissues. Limited evidence: Assigned when the overall evidence base (across each KCC) was suggestive, with studies covering a narrow range of experiments, endpoints, and/or species. Also assigned because of inconsistencies in similar studies, and /or incoherence across studies of different endpoints or different experimental systems. Inadequate evidence: Assigned when there were few or no data available, or when there were unresolved questions about the adequacy of the study design, conduct, or interpretation, or the results were negative. [2]Overall strength of mechanistic activity was evaluated by examining the robustness of the data within each KCC, as well as identifying individual KCCs considered to have strong findings, or the combination of two of more KCCs.*Overall KCC10 was considered limited based on studies of cell proliferation that supported mechanisms the drive tumorgenicity that would not operate in humans (see Table 5).

post oral administration of low dose levels (0.00186 to 11.9 mg/kg-bw) of ^{14}C-MTBE to male mice. Du et al. (2005) used doubly radiolabeled MTBE ($CH_3CO^{14}_3CO^{14}CH_3$) to identify DNA adducts in these same tissues following a single low oral dose of radiolabeled MTBE to mice (0.00095 – 6.18 mg/kg-bw), with evaluation at up to 500 h post-administration. A dose-dependent increase in DNA adducts were detected in lung and liver, with peak levels at 12 and 6 h, respectively, with the level decreasing back to baseline by five days in both tissues. The analytical method used for measuring these adducts was accelerated mass spectroscopy. Bus et al., (2022) identified that without use of a synthetic standard, it is unclear if these results reflect labeled DNA adducts or metabolic incorporation of the ^{14}C label into DNA through cellular carbon pools generated from the metabolism of ^{14}C-MTBE (e.g., formation of ^{14}C-formate). Neither Yuan et al., (2007) nor Du et al. (2005) used a standard to distinguish DNA adduct formation from incorporation of ^{14}C into carbon pool, as such, these results are not considered reliable for considering the strength of activity within this KCC.

Overall, the strength of the evidence for KCC1, "*Is electrophilic or can be metabolically activated to an electrophile*", is 'Limited' based on no data in exposed humans or human primary cells or tissues, not reliable study data in animal models, and activity of DNA-Protein crosslinks measured in non-human mammalian models *in vitro* (Fig. 4).

KCC2 "Is genotoxic" (Appendix A. Supplementary data, Table S3)

In vitro non-mammalian mutagenicity (Table 2a). *In vitro* mutagenicity (Ames) activity across several publications is consistently negative with exposure to MTBE with and without metabolic (S9) activation (ARCO Chemical, 1980; Kado et al., 1998; Zhou and Ye, 1998; McGregor et al., 2005). *Salmonella typhimurium* strains (TA90, TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538) were tested at concentrations up to 7,400 mg/assay. Although, one study reported a weak mutagenic response observed in TA102 with and without S9 activation (Williams-Hill et al., 1999), this study was considered unreliable based on reporting concerns (Klimisch Score = 3). McGregor et al. (2005) reevaluated the mutagenicity of MTBE in *Salmonella typhimurium* strains TA102 and found no activity with and without metabolic activation. MTBE induced forward mutations in the presence of rat S9 activation in a modified mouse lymphoma assay (exposure concentrations of 1–4 mL/mL [8.39 to 33.6 mM]) was conducted to distinguish whether the mutagenicity could be attributed to formaldehyde formed from the metabolism of MTBE. The results showed that mutagenicity was due to the formaldehyde formed during S9 activation (Mackerer et al., 1996).

In vitro primary human cells (Table 2b). Data for genotoxicity endpoints in primary human cells were limited to a single study. In this study, human lymphocytes exposed to MTBE concentrations up to 200 μM, showed an increase in double and single-stranded DNA breaks at the highest tested concentration (Chen et al., 2008). Overall, this assay was considered reliable since no concerns were identified for study design or reporting. However, it is noted that activity in this assay was inconsistent with the lack of activity reported across *in vitro* assays in non-human mammalian cell lines or primary cells exposed to a similar MTBE concentration.

In vitro non-human mammalian primary cells or cell lines (Table 2b). In this category, many of the assays were not considered reliable. However, two reliable genotoxicity studies were conducted in mammalian (non-human) cell lines in which one of the studies showed a significant increase in DNA double strand breaks observed in an enhanced comet assay using a rat fibroblast cell line (Rat-1) following exposure to a single MTBE concentration (0.84 mM) for up to 12 h (Sgambato et al., 2009). The other reliable study measured chromosomal aberrations in CHO cells exposed to MTBE concentrations up to 5 μL/mL in which clastogenic activity was not observed with or without metabolic activation (ARCO, 1980). Assays not considered to be reliable, either due to endpoint such as unscheduled DNA synthesis (UDS) (currently not being used in genotoxicity evaluations), assays not incorporating measures of cell viability, or using recommended number of cells to evaluate DNA damage/strand breaks, were not considered (Song et al., 2002; Yang et al., 2005).

In vivo mammalian studies (Table 2c). *In vivo* reliable studies in experimental animal models reported consistently negative data for genotoxic and mutagenic endpoints (ARCO, 1980; Vergnes et al., 1989;1993; McKee et al., 1997; Kado et al. 1998; Gollapudi et al., 2024) with only one study, not considered reliable, showing activity (chromosomal aberrations) (Darwish and Mosallam, 2019). *In vivo* studies are more useful in assessing the potential mutagenic and genotoxic potential of chemicals considering metabolism is ongoing. This is especially true for MTBE since the metabolites identified in both human and animal studies are the same. Several studies reported negative micronucleus formation in the bone marrow in MTBE exposed mice. This included male and female mice administered a single intraperitoneal MTBE injection at doses ranging from 0.25 to 1.75 g/kg-bw (Kado et al., 1998), in mice exposed via inhalation to 400, 3000, 8000 ppm (6 h/day for 2 days) (Vergnes et al., 1989), and in mice exposed to MTBE via inhalation for 2 days (6 hr/day) (McKee et al., 1997). In a recent inhalation study reported in transgenic male F344 Big Blue rats exposed to 0, 400, 1000, 3000 ppm MTBE for 28 days (6 h/day), no changes in the mutant frequencies at the *cII* locus of the transgene in liver, bone marrow, kidney, or nasal epithelium were reported by Gollapudi et al., 2024, confirming the lack of mutagenicity of MTBE following high concentrations of exposure. Not only is this finding critical since it was conducted *in vivo* in an animal model capably of metabolizing MTBE, a positive control was run simultaneously to confirm a negative finding within tissues.

Overall, the strength of the evidence for KCC2, "*Is genotoxic*", is 'Limited' based on a single *in vitro* study in primary human lymphocytes that reported evidence of DNA damage, a lack of activity in non-human mammalian and nonmammalian *in vitro* assays, as well as in animal *in vivo studies* (Fig. 4).

KCC3 "Alters DNA repair or causes genomic instability" Supplemental File C (Appendix A. Supplementary data Table S4). The endpoint mapped to KCC3 measured in two reliable studies was 8-oxoguanine glycosylase (OGG1), a base excision enzyme responsible for removing 8-oxoguanine (an oxidized DNA modification) (Li et al., 2008; 2009). In the first study, the only change was a decrease in OGG1 expression in the testes of rats administered 1,600 mg/kg-bw/day MTBE by oral gavage for two weeks. There was no change in expression of OGG1 following four weeks of MTBE administration at this same dose level of 1,600 mg/kg-bw/day (Li et al., 2008). In an *in vitro* study of rat primary Sertoli cells exposed to 5,000 μM MTBE for up to 48 h, no effect on OGG1 expression was observed (Li et al., 2009).

Overall, the strength of evidence for KCC3 "*Alters DNA repair and genomic instability*" is considered 'Limited' for evaluating the effects of MTBE exposure (Fig. 4).

KCC4 "Induces epigenetic alterations" Supplemental File C (Appendix A. Supplementary data Table S5). Of the available data on epigenetic alterations, only one *in vitro* study, conducted in a mouse hippocampal neuronal cell line (HT22), was reliable (Ma et al., 2017). Authors reported increased gene expression and protein levels for SIRT, a NAD + -dependent histone deacetylase, at 0.125 mmol/L MTBE. Changes in gene expression and protein levels were also reported at higher concentrations of MTBE, however these authors reported a significant decrease in cell viability starting at 0.25 mmol/L.

Both an *in vivo* study in Wistar rats and an *in vitro* study in HepaRG cells was conducted to assess changes following exposure to MTBE. Rats were administered MTBE by oral gavage for 24 weeks at (0, 0.1, 1, 100 mg/kg-bw/day). A significant decrease in miR-18a-5p and miR-193 at all dose levels was reported, with no significant change for miR-214 and miR-140. *In vitro* changes in HepaRG cells were only observed at

cytotoxic concentrations and were, therefore considered unreliable (Wang et al., 2025).

Rota et al., (2018) reported a significant association between alterations in DNA methylation of Alu-YD6 and L1-Ta (transposable elements) in blood lymphocytes and urinary MTBE levels in humans exposed to MTBE through occupational exposure (i.e. petrol station workers). However, this study was not considered reliable due to lack of consideration of confounding co-exposures in their analysis such as benzene, which was also measured, as well concerns regarding selection bias and cross-sectional study design. Additional details on rationale for study quality assessment of Rota et al., can be found in Appendix A. Supplementary data, Table S5.

Overall, the strength of evidence for KCC4 '*Induces epigenetic alterations*' is considered 'Limited' based on data available in one reliable study *in vitro* and one *in vivo* study in rats (Fig. 4).

KCC5 "Induces oxidative stress" (Appendix A., Supplementary data Table S6). As noted by Bus et al., 2017, oxidative stress is widely recognized to be more commonly associated with non-cancer disease outcomes. In many cases for this KCC the use of specific methodology was found not reliable for assessing a particular endpoint and assigned a Klimisch score of 3 (not reliable) based on review of reliability measures for oxidative stress reported by Halliwell and Whiteman (2004) and Murphy et al., (2022). Because so many of these endpoints were considered active, they are included in Table 3 for transparency, however, studies that used methods that were not reliable were not used for integration to determine overall activity.

In vitro human and non-human mammalian assays (Table 3a). Measures of oxidative stress were evaluated in human primary cells *in vitro*, along with *in vitro* and *in vivo* animal models. These measures included oxidative DNA damage markers (8-OHdg levels, 8-OH2dg adducts, free-radical induced breaks), lipid peroxidation (malondialdehyde (MDA) levels/activity), antioxidant levels/activity (GSH, SOD, NQO, GPx, CAT, total antioxidant capacity), or ROS levels. Method limitations or potential artifacts (e.g., measurement interference), are summarized for each assay. Several reliable studies were reported KCC5 endpoints in mammalian cell lines and primary cells. Most of these reported changes in antioxidant enzyme expression or activity (e.g., SOD, SOD_{EX}, GSH, glutathione peroxidase (GSH-PX), or glutathione disulfide (GSSG)) and oxidative DNA damage markers (8-OHdg, OGG1) (Xie et al., 2017; Li et al., 2007; Li et al., 2009; Ma et al., 2017, Sgambato et al., 2009).

Mammalian in vivo studies (Table 3b). Five reliable studies measured oxidative stress endpoints in mammalian *in vivo* models; results for these studies were inconsistent. In one study, juvenile BALB/c male mice exposed to MTBE via drinking water (0, 80, 800, 8000 ppb for 51-days) showed no change in lipid peroxidation (malondialdehyde) or markers of oxidative DNA damage (8OH2dG) in liver (de Peyster et al., 2008). Another study reported increased oxidized LDL levels (indicator for lipid peroxidation) at all dose levels administered to Wistar rats (0, 0.01, 1, 100 mg/kg-bw/day for 24 weeks (Wang et al., 2025). In male Sprague-Dawley rats administered 0, 400, 800 or 1600 mg/kg-bw/day MTBE for two or four weeks by oral gavage, gene expression changes that suggested oxidative stress in the testes of rats administered at the high dose of 1600 mg/kg-bw/day MTBE for two weeks, but not at 4 weeks. The genes analyzed included extra-cellular superoxide dismutase (SOD_{EX}) and 8-oxoguanine DNA glycosidase (OGG1), markers of oxidative stress (i.e., antioxidant enzymes and oxidative DNA damage base excision enzymes) (Li et al., 2008). Decreased glutathione (GSH) was detected in liver of male Sprague-Dawley rats exposed to MTBE in drinking water (1000 μg/L) for 90 days (Saeedi et al., 2017b). In two reliable studies, also conducted in rats, antioxidant enzymes were examined and showed no effects on SOD or glutathione dismutase (GPX) activity in serum or quinone oxidoreductase (NQO1) activity in liver (Elovaara et al., 2007; Khalili et al., 2015).

Overall, the strength of evidence for KCC5 '*Induces oxidative stress*' is considered 'Limited' based on the inconsistent activity in animal models

Table 2a
In vitro non-mammalian and mammalian mutagenicity assays* *(Documentation of all data provided in Supplemental File C; Table S3).*

Assay Endpoint	Test System (species, strain)	Concentration of MTBE[a]	Results		Comments/ Quality Assessment	Reference
			w/o metabolic activation	w/ metabolic activation		
Modified Ames (microsuspension)	*S. typhimurium TA1535, TA98, TA100, TA104*	0, 30, 90, 300, 925, 1850, 3700, 7400 mg/tube	–	–	Overt toxicity observed at 7400 μg/plate at all S9 concentrations: S9 concentration of 300, 600, 1200 mg/mL	Kado et al., 1998
Ames Assay	*S. typhimurium TA100, TA98*	0, 25, 50, 100 mg/plate	–	–	NC	Zhou et al., 2000a
Ames Assay	*S. typhimurium TA100, TA98*	0, 25, 50, 100 mg/plate	–	–	NC	Zhou and Ye, 1998
Ames Assay	*S. typhimurium TA102*	0,0.5,0.75, 1.5, 2.5 mg/plate	W+	W+	Approximate concentrations taken from graph. Weakly positive response observed, with human and rodent S9 remaining within 2-fold of control and considered inconclusive. **Klimisch 3, included based on weak activity**	Williams-Hill et al., 1999
Ames Assay	*S. typhimurium TA102*	0, 100, 200, 500, 1000, 2500, 5000 mg/plate	–	–	NC	McGregor et al., 2005
Ames Assay	*S. typhimurium TA-1535, TA1537, TA-1538, TA90, TA100*	0, 0.01,0.1,1, 5 and 10 μL/plate	–	–	NC	ARCO Chemical, 1989
Modified mouse lymphoma assay; mutations frequency	L5178Y tk-/-	0,1, 2, 3, 4 μL/mL	+	–	Induction of mutations frequency, controlled for volatility of MTBE	Mackerer et al., 1996

*All assays with Klimisch Scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.
Abbreviations.
W+, weakly positive.
–, no activity.
NC, no comment.
[a] Doses/concentrations with significant effects are bolded.

Table 2b
In vitro human and non-human mammalian genotoxicity assays* *(Documentation of all data provided in Supplemental File C; Table S3).*

Assay Endpoint[a]	Species, Tissue/Cell Line	Concentration of MTBE[b,c]	Results	Comments/Quality Assessment	Reference
In Vitro Human					
Double-strand DNA break	Primary Human Lymphocytes	0, 50, 100, **200 µM**	+	NC	Chen et al., 2008
Single-strand DNA break	Primary Human Lymphocytes	0, **200 µM**	+	NC	Chen et al., 2008
In Vitro Non-Human Mammalian					
DNA strand breaks (single-cell gel electrophoresis)	Rat, Alveolar type II cell suspension	0, 0.025, **0.05, 0.2** mM	+	Scored 25 cells vs. 50 recommended by Tice et al., 2000. Cells were exposed for only 1 h, vs. the 3–6 h recommended by the International Workshop on Genotoxicity Test Procedures. **Klimisch 3; included based on activity**	Yang et al., 2005
DNA Strand Breaks (Comet Assay)	Mouse, L-929	0, 9.375, 18.75, **37.5, 75, 150** mg/mL [102–1700 mM]	+	The comet rate increased from 4 % to 85 % as the concentration of MTBE increased from 9.375 to 150 mg/mL. Unreliable results due to high concentrations tested without testing cytotoxicity. **Klimisch 3; included based on activity**	Song et al., 2002
DNA strand breaks (modified comet assay)	Rat-1	0, **0.84** mM	+	Not all concentrations tested were reported.	Sgambato et al., 2009

*All assays with Klimisch Scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.
Abbreviations:
W+, weakly positive.
−, no activity.
+ positive activity.
NC, no comment.
[a] Unscheduled DNA synthesis (UDS) assays were excluded based on this assay not currently being used in regulatory assessments.
[b] Doses/concentrations with significant effects are bolded.
[c] Concentrations in brackets were calculated as molarity.

Table 2c
In vivo mammalian genotoxicity and mutagenicity studies* (Documentation of all data provided in Supplemental File C; Table S3).

Assay Endpoint[a]	**Species, Tissue**	**MTBE Dose**[a,c]	**Dose Regimen (route); Study Duration**	**Results**	**Comments/Quality Assessment**	**Reference**
Chromosomal Aberrations (CA)	Fischer 344 Rat, Bone Marrow	0, 800, **1600** mg/kg	Oral Gavage; 14, 28 days	+/-	Increased CA after 14 days exposure at highest dose; no significant effects at 28-day exposure Method states 50 metaphases evaluated, whereas Table 1 states 300 metaphases evaluated: inconsistent reporting, unable to evaluate. **Klimisch = 3**	Darwish and Mosallam, 2019
Chromosomal Aberrations (CA)	Rat, Bone Marrow	0, 800, 4000 and 8000 ppm [800–8000 mg/kg]	Inhalation; 6 h/d for 5 days	–	NC	Vergnes et al., 1989
Micronucleus	CD-1 Mouse, Bone Marrow	0, 400, 3000, 8000 ppm [400–8000 mg/kg]	Inhalation; 6 h/day for 2 days	–	NC	Vergnes et al., 1993
Micronucleus	CD-1 Mouse, Bone Marrow	0, 800, 4000, 8000 ppm [800–8000 mg/kg]	Inhalation; 6 h/day for 2 days	–	NC	Mckee et al., 1997
Micronucleus	Mouse, Bone Marrow	0, 0.25, 0.5, 1, 1.5, 1.75 g/kg-bw [250–1750 mg/kg-bw]	IP injection; single dose	–	NC	Kado et al., 1998
DNA breaks (Comet assay)	Kunming mice, Liver, Kidney, Lung	0, **108**, **1440,** and **4968** mg/m^3 [0.146–6.71 mg/kg]	Inhalation; 4 h/day for 20 days	+	Scored 25 cells vs. 150 recommended in OECD 489. Scoring was manual, rather than automated as recommended in TG 489. Scoring was not blinded and did not include an assessment of number of hedgehogs or "non-scorable" (recommended TG 489) **Klimisch = 3**	Yang et al., 2005
Mutant Frequency	Fischer 344 Big Blue Rat, Nasal, Liver, Kidney, and Bone Marrow	0, 400, 1000, 3000 ppm [400–3000 mg/kg]	Inhalation; 6 h/day for 28 days	–	OECD TGR (TG 488) compliant study.	Gollapudi et al., 2024

*All assays with Klimisch scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.
Abbreviations.
NC, no comment.
+ positive activity.
− no activity.
+/-, equivocal activity.
[b]Doses/concentrations with significant effects are bolded.
[a] Unscheduled DNA synthesis (UDS) assays were excluded based on this assay not currently being used in regulatory assessments.
[c] Concentrations in brackets were calculated as mg/kg.

Table 3a
In vitro human and mammalian oxidative stress endpoints* (Documentation of all data provided in Supplemental File C; Table S6).

Assay Endpoint	Species/Cell line, Tissue	MTBE Concentration[a,b]	Results	Comments/SME Assessment	Reference
Reactive Oxygen Species (ROS) Levels	Human Hemoglobin	0, 100, 200, 300, 400, 500 μM	+	No cytotoxicity measured; **Klimisch = 3**	Najdegerami et al., 2017
Reactive Oxygen Species (ROS) Levels	Human Primary Lymphocytes	0, 0.1, 0.5, 1 mM	+	Increased ROS at 30–180 mins; Cytotoxicity observed at all doses at 360 mins; Klimisch = 3[c]	Salimi et al., 2016
Lipid Peroxidation (MDA levels)	Human Primary Lymphocytes	0, **0.1, 0.5, 1** mM	+	Increased peroxidation at 180 mins; cytotoxicity observed at all doses at 360 mins, **Klimisch = 3**[d]	Salimi et al., 2016
Lipid Peroxidation (MDA levels)	Human Bronchial Epithelial Cell Line (16HBE)	0, 50, 100 mM	–	No cytotoxicity measured; **Klimisch = 3**	He et al., 2021
Antioxidant Enzyme (GSH; GSSG)	Human Primary Lymphocytes	0, **0.1, 0.5, 1** mM	+	Decreased GSH levels; Increased GSSG levels at 180 mins; cytotoxicity observed at all doses at 360 mins, **Klimisch = 3**	Salimi et al., 2016
Antioxidant Enzyme (GSH-PX, SOD, CAT)	Human Bronchial Epithelial Cell Line (16HBE)	0, 50, 100 mM	–	No cytotoxicity measured; **Klimisch = 3**	He et al., 2021
Antioxidant Enzyme (SODex)	Rat Primary Sertoli Cells	0, 0.5, 50, **5000** μM	+	Effects observed at cytotoxic doses. Significant decrease in cell viability at 5,000 μM at 6 and 12 h. **Klimisch = 3**	Li et al., 2009
Antioxidant Enzyme (SOD, GSH-PX, CAT)	Chinese Hamster Ovary (CHO)	0, 0.5, **5.0, 25.0, 50.0, 100.0** mM [500–100,000 μM]	+	NC	Xie et al., 2017
Antioxidant Enzyme (cytosolic SOD, SODex activity)	Rat Primary Sertoli Cells	0, 0.5, **50 μM, 5 mM** [5,000 μM]	+	Effects observed at 6–18 h. Decreased cell viability at 5 mM at 18 h	Li et al., 2007
Antioxidant Enzyme (SOD)	HT22 Mouse	0, 0.125, 0.25, **0.5, 1.0, 2.0** mmol/L [125–2,000 μM]	+	Effects observed at cytotoxic doses. Cytotoxicity observed from 0.25 to 2.0 mmol/L. **Klimisch = 3**	Ma et al., 2017
Antioxidant Enzyme (GSH/T-GSH; GSSG)	HT22 Mouse	0, **0.125, 0.25, 0.5, 1.0, 2.0** mmol/L [125–2,000 μM]	+	Cytotoxicity observed from 0.25 to 2.0 mmol/L. **Klimisch = 3**	Ma et al., 2017
Lipid Peroxidation (MDA levels)	Chinese Hamster Ovary (CHO)	0, 0.5, **5.0, 25.0, 50.0, 100.0** mM [500 – 100,000 μM]	+	**Klimisch = 3**[d]	Xie et al., 2017
Lipid Peroxidation (MDA levels)	Rat Primary Sertoli Cells	0, 0.5, 50, **5000** μM	+	Effects observed at cytotoxic doses. Significant decrease in cell viability at 5,000 μM at 6 and 12 h; **Klimisch = 3**[d]	Li et al., 2009
Lipid Peroxidation (MDA levels)	HT22 Mouse	0, 0.125, **0.25, 0.5, 1.0, 2.0** mmol/L [125–2,000 μM]	+	Effects observed at cytotoxic doses. Cytotoxicity observed from 0.25 to 2.0 mmol/L; **Klimisch = 3**[d]	Ma et al., 2017
Lipid Peroxidation (MDA levels)	Rat Primary Sertoli Cells	0, **0.5**, 50 uM, 5 mM [5,000 μM]	+	Effects observed at 18 h. Effects observed at 6–18 h. Decreased cell viability at 5 mM at 18 h; **Klimisch = 3**[d]	Li et al., 2007
Oxidative DNA Damage (OGG1 expression)	Rat Primary Sertoli Cells	0, 0.5, 50, 5000 μM	–	Significant decrease in cell viability at 5,000 at 6 and 12 h.	Li et al., 2009
Oxidative DNA Damage (8-OHdg levels)	Rat-1, Fibroblast	0, **0.84** mM [840 μM]	+	Measured immunohistochemically—reliable measure	Sgambato et al., 2009
Reactive Oxygen Species (ROS) Levels	HT22 Mouse	0, **0.125, 0.25, 0.5, 1.0, 2.0** mmol/L [125–2,000 μM]	+	Cytotoxicity observed from 0.25 to 2.0 mmol/L; Increased ROS at 30–180 mins; Cytotoxicity observed at all doses at 360 mins; **Klimisch = 3**[c]	Ma et al., 2017
Reactive Oxygen Species (ROS) Levels	Rat Primary Sertoli Cells	0, **0.5, 500, 5,000** μM	+	Effects observed at cytotoxic doses. Significant decrease in cell viability at 5,000 at 6 and 12 h; **Klimisch = 3**[c]	Li et al., 2009
Reactive Oxygen Species (ROS) Levels	Rat Primary Sertoli Cells	0, 0.5, **50 uM, 5 mM** [5,000 μM]	+	Effects observed at 1–18 h. Decreased cell viability at 5 mM at 18 h; Increased ROS at 30–180 mins; Cytotoxicity observed at all doses at 360 mins; **Klimisch = 3**[c]	Li et al., 2007
Reactive Oxygen Species (ROS) Levels	Rat Primary Immature Leydig Cells	0, 100, **200, 300** mM	+	**Klimisch = 3**[c]	Zhu et al., 2022

* All assays with Klimisch scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC Strength.
Abbreviations:
IP = intraperitoneal.
+ positive activity.
– negative activity.
+/- equivocal.
NC; no comments.
[a] Doses/concentrations with significant effects are bolded.
[b] Concentrations in brackets were calculated as molarity.
[c] The substrate DCF is sensitive to local O_2 levels and pH, and fluorescence yield may not be linear with increased ROS levels (Murphy et al., 2022). High light intensity may contribute to photochemical oxidation to fluorescent products. DCF can also undergo extracellular reactions (Halliwell et al., 2004).
[d] TBARS nonspecific method, because thiobarbituric acid (TBA) generates chromogens and many biomolecules other than MDA. The method is not recommended for evaluation of oxidative lipid damage because of the low specificity that can result in false-positive results (Murphy et al., 2022).

Table 3b
In vivo mammalian oxidative stress endpoints* (Documentation of all data provided in Supplemental File C; Table S6).

Assay Endpoint	Species/Cell Line, Tissue	MTBE Dose[a,b]	Dose Regimen (route; study duration)	Results	Comments/SME Assessment	Reference
Antioxidant Enzyme (SOD)	Sprague-Dawley Rat, Testes	0, 400, 800, **1600** mg/kg	Oral Gavage; 2,4 weeks	+	NC	Li et al., 2008
Antioxidant Enzyme (SOD and GPX)	Sprague-Dawley Rat, Serum	0, 400, 800, 1600 mg/kg	Oral Gavage; 30 days	–	NC	Khalili et al., 2015
Antioxidant Enzyme (NQO1)	Wistar Rat, Liver	0, 13.5, 20 mmol/kg [1,190 –1,763 mg/kg]	Oral Gavage; 3 days	–	NC	Elovaara et al., 2007
Antioxidant Enzyme (GSH Content)	Sprague-Dawley Rat, Liver	0, **40, 200, 1000** ug/L [0.04–1 mg/kg]	Drinking Water; 90 days	+	NC	Saeedi et al., 2017
Lipid Peroxidation (MDA levels)	Sprague-Dawley Rat, Liver	0, 40, **200, 1000** ug/L [0.04–1 mg/kg]	Drinking Water; 90 days	+	**Klimisch = 3**[c]	Saeedi et al., 2017
Lipid Peroxidation (MDA levels)	BALB/c Mouse, Liver	0, 80, 800, 8000 ppb [80–8000 mg/kg]	Drinking Water; 51 days	–	Reliable measure of MDA using HPLC	De Peyster et al., 2008
Lipid Peroxidation (MDA levels)	Sprague-Dawley Rat, Testes	0, 400, 800, **1600** mg/kg	Oral Gavage; 2, 4 weeks	+	**Klimisch = 3**[c]	Li et al., 2008
Lipid Peroxidation (MDA levels)	Kunming Mouse, Kidney	0, 108, **1440, 4968** mg/m^3	Inhalation; 4 hrs/day for 20 days	+	Effects observed in female mice (1440 and 4968 mg/m^3), male mice (4968 mg/m^3). **Klimisch = 3**[c]	Yang et al., 2005
Lipid Peroxidation (MDA levels)	Mouse, Liver	0, **50**, 200, **500** mg/kg	IP injection; Single Dose	+	**Klimisch = 3**[c]	Katoh et al., 1993
Lipid Peroxidation (MDA levels)	Mouse, Liver	0, 50, **200** mg/kg	Inhalation; 5x/week for 1 week or 4 weeks	+	**Klimisch = 3**[c]	Katoh et al., 1993
Lipid Peroxidation (Oxidized LDL)	Wistar Rat, Blood	0, 1, 100 mg/kg/day	Oral Gavage, 24 weeks	+	NC	Wang et al., 2025
Reactive Oxygen Species (ROS) Levels	NMRI Mouse, Liver and Brain	0, **500, 1000** mg/kg bw/day	IP Injection; 1 day	+	**Klimisch = 3**[d]	Faizi et al., 2020
Oxidative DNA Damage (8OH2dG adducts)	BALB/c Mouse, Liver	0, 80, 800, 8000 ppb [80–8000 mg/kg]	Drinking Water; 51 days	–	Study used HPLC—reliable measure, although spurious oxidation may occur—best method is LC-MS/MS or UPLC-MS/MS.	De Peyster et al., 2008
Oxidative DNA Damage (OGG1 expression)	Sprague-Dawley Rat, Testes	0, 400, 800, **1600** mg/kg	Oral Gavage; 2, 4 weeks	+	NC	Li et al., 2008
Total Antioxidant Capacity (TAC)	BALB/c Mouse, Liver	0, 80, 800, 8000 ppb [80–8000 mg/kg]	Drinking Water; 51 days	–	**Klimisch = 3**[e]	De Peyster et al., 2008
Total Antioxidant Capacity (TAC)	Sprague-Dawley Rat, Testes	0, **400, 800**, **1600** mg/kg	Oral Gavage; 2, 4 weeks	+	**Klimisch = 3**[e]	Li et al., 2008

*All assays with Klimisch scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.

[a] Doses/concentrations with significant effects are bolded.

[b] Concentrations in brackets were calculated as mg/kg.

[c] TBARS nonspecific method, because thiobarbituric acid (TBA) generates chromogens and many biomolecules other than MDA. This is not recommended for evaluation of oxidative lipid damage because of the low specificity that can result in false positive results (Murphy et al., 2022).

[d] DCF is sensitive to local O_2 levels, pH, and florescence yield may not be linear with increased ROS levels (Murphy et al., 2022); DCF can undergo extracellular reactions or photochemical oxidation to fluorescent products (Halliwell et al., 2004).

[e] TAC changes may differ based on blood chemistry, as well as diet, and should be interpreted with caution (Halliwell et al., 2004).

and primary human cells. In non-human mammalian cell lines and primary cells, antioxidant enzyme and oxidative DNA damage markers were consistently positive, but over a narrow range of endpoints and species (Fig. 4).

KCC6 "Induces chronic inflammation" (Appendix A. Supplementary data, Table S7). Evaluation of MTBE in a human cell line (THP-1) reported no effect on inflammatory markers (TNF-α, IL-1B) following exposure at concentrations ranging from 1-10 mM (Ren et al., 2021). A significant increase in TNF- α protein levels starting at 1 mg/kg-bw/day and gene expression of TNF- α and IL-1B starting at 0.01 mg/kg-bw/day was reported in Wistar rats administered MTBE at 0, 0.1, 1, and 100 mg/kg-bw/day by oral gavage (Wang et al., 2025). Two reliable *in vivo* drinking water (DW) studies were conducted in male rats that evaluated microscopic tissue changes or markers of inflammation (Saeedi et al., 2017a; Saeedi et al., 2017b). In one study, histopathological examination of liver revealed inflammation that occurred with increased severity observed at MTBE concentrations of 200 and 1000 μg/L (following 90 days exposure). The severity score was mild following MTBE exposure, versus absent in controls and at 40 μg/L MTBE. In the second publication, increased serum C-reactive protein levels were observed at all MTBE DW exposure concentrations (40, 200, and 1000 μg/L) (Saeedi et al., 2017a; Saeedi et al., 2017b).

Overall, the strength of evidence for KCC6 "I*nduces chronic inflammation*" is considered 'Limited' based on lack of findings in exposed humans and human primary cells or tissues, with several studies in other model categories with minimal activity (Fig. 4).

KCC7 "Is immunosuppressive" Supplemental File C (Appendix A. Supplementary data, Table S8). Five reliable studies in rodents examined changes in white blood cell counts following *in vivo* MTBE exposure to assess its potential immunosuppressive properties. Decreased white blood cell counts were observed in three studies (Badr et al., 2019; Katoh et al., 1993; Robinson et al., 1990), including one in male mice, administered a single MTBE dose (via i.p. injection). However, a decrease in leukocyte (white blood cell) numbers occurred in mice 24 h post- a single dose of 500 mg/kg-bw, but not in mice administered 50 or 200 mg/kg-bw MTBE, even when administered for a longer period of five days (Katoh et al., 1993). In rats administered low (1 mg/kg-bw/day for 10 days) or high MTBE doses (1,200 mg/kg-bw/day for 90 days) via oral gavage, the white blood cell count was significantly reduced (Badr et al., 2019; Robinson et al., 1990). Dose levels of 10 – 1,428 mg/kg-bw/day for intermediate time periods (14–28 days), however, produced no effects (Badr et al., 2019; Robinson et al., 1990). Two MTBE inhalation exposure studies in rats were conducted, with one study showing no change in white blood cell counts (Greenough et al., 1980) and no IgM

antibody-forming cell responses (White et al., 2014) with exposure up to 20,000 mg/m^3.

Overall, the strength of evidence for KCC7, "*Is immunosuppressive*", is 'Limited' based on inconsistent results across similar animal study designs with a narrow range of endpoints, as well as no data in exposed humans or human primary cells or tissues (Fig. 4).

KCC8 "Modulates receptor-mediated effects" (Appendix A. Supplementary data, Table S9). For endpoints that reflect receptor mediated effects, changes associated with individual nuclear receptors associated with carcinogenic outcomes are reviewed by receptor.

Androgen receptor (AR) mediated effects. MTBE did not show activity *in vitro* in assays where cytotoxicity was evaluated. In isolated Leydig cells from Sprague Dawley rats, exposure to MTBE (50 or 100 mM) resulted in a reduction in testosterone levels (de Peyster et al., 2003). MTBE did not influence steroidogenesis (testosterone synthesis) in H295R cells at concentrations of 0.0001–100 μM and was negative when tested at concentrations up to 1 mM in a cell-free assay with the AR isolated from rat prostates (de Peyster et al., 2014). In addition, using recombinant human liver microsomes, MTBE did not inhibit aromatase activity at concentrations up to 1 mM (de Peyster et al., 2014).

A total of six reliable studies in mammalian experimental models reported MTBE exposure effects on endpoints that reflect androgen pathway modulation. MTBE effects on serum hormone levels were reported in all six studies. In young male rats, decreased serum testosterone was observed following two weeks of administration of high MTBE doses levels (800 or 1,600 mg/kg-bw/day) by oral gavage, but after four weeks of exposure, testosterone levels were increased (Li et al., 2008). In three other studies conducted in adult male rats, exposure durations of 15-, 21- or 28-days led to reduced serum and interstitial fluid testosterone levels, with increased serum dihydrotestosterone levels following oral gavage of MTBE at dose levels up to 300 mg/kg-bw/day (Williams et al., 2000; Khalili et al., 2015; Zhu et al., 2022). In one study, this effect was shown not to be persistent, and the reduction of serum or interstitial fluid testosterone was at control levels by 28 days (Williams et al., 2000). In two studies, where adult male rats administered MTBE at high dose levels (up to 2,000 mg/kg-bw/day), changes in serum testosterone or testosterone levels in testicular homogenates were not reported (de Peyster et al., 2008; de Peyster et al., 2014). MTBE also did not inhibit aromatase activity when measured in liver and testis microsomes isolated from rats administered up to 1,200 mg/kg-bw/day MTBE for 14 days (de Peyster et al., 2014). Despite some signals of testosterone changes, overall, hormone changes across these studies were not consistent or persistent following administration of high MTBE doses.

Estrogen receptor (ER) mediated effects. In a competitive binding cell-free assay with recombinant human ER, MTBE was found to be negative at concentrations up to 0.1 mM (Moser et al., 1998). MTBE had no effect on steroidogenesis (estradiol synthesis) in H295R cells at concentrations of 0.0001–100 μM (de Peyster et al., 2014).

MTBE exposure did not affect serum estradiol or estrogen levels measured in rats (400–12,000 mg/kg-bw/day) and mice (1,800 mg/kg-bw/day or 80–8,000 ppm) (de Peyster et al., 2014; de Peyster et al., 2008; Moser et al., 1998), with no ER immunoreactivity observed in the uterus, cervix, and vagina of exposed mice (Moser et al., 1998).

The ability of MTBE to bind or activate the ER and AR was predicted using QSAR toolbox (Version 4.7). The results are provided in Supplemental File C**, Table S13**. MTBE was predicted to be negative in all the AR antagonist QSAR models. However, MTBE was outside of domain for the ER activation models which included 2 sets of ER binding models. MTBE was negative in 3 of 4 ER models where the full training set was used and was out of domain when the "balanced training set was used. Overall, these data imply that MTBE most likely does not bind or activate these receptors, as also reported using the COMPARA and CERAPP models for AR and ER, respectively, as reported on EPA's CompTox Chemicals Dashboard (EPA, 2024b).

Thyroid receptor and thyroid pathway key events. A reliable study in male Sprague-Dawley rats administered MTBE by oral gavage (0, 250, 500, 1000, or 1500 mg /kg-bw/day) for 15 or 28 consecutive days showed no changes at 15 days with a decrease in serum triiodothyronine (T3) at 28 days without changes in thyroxine (T4) or thyroid stimulating hormone (TSH) (Williams et al., 2000).

Other receptor mediated effects. Based on the influence of CAR/PXR induction on phase 1 drug metabolizing enzyme such as cytochrome P450, changes in enzyme levels and activity associated with these enzymes are included under KCC8. One study measured alterations in PXR gene expression and protein levels and downstream targets *in vivo* in the liver of Wistar rats administered MTBE by oral gavage (0, 0.01, 1, 100 mg/kg-bw/day) and *in vitro* in a human liver cell line (HepaRG) at 0, 100 mmol/L (Wang et al., 2025). A statistically significant increase in PXR protein levels and expression were reported *in vitro* and *in vivo*. However, *in vitro* results were not considered reliable due to effects only being observed at cytotoxic doses (Supplemental File C, **Table S9**). Three *in vivo* studies measured P450 induction in the liver and testis of rats administered equivalent doses of MTBE that ranged between 250 and 1800 mg/kg-bw/day by oral gavage or exposed by inhalation to up to 8,000 ppm. In one study, no induction of P450 enzymes occurred in the liver or testis following 14 days exposure (de Peyster et al., 2014). The remaining studies reported an increase in P450 content in the liver following a 3- or 21-day exposure (Moser et al., 1996a; Moser et al., 1996b) with P450 induction (increased enzyme concentration) observed at 15 and 28 days (Williams and Borghoff, 2000). Also reported was an increased hepatic enzyme activity (EROD and PROD) in the liver of mice exposed to MTBE (Moser et al., 1996a; Moser et al., 1996b).

Overall, the strength of the evidence for KCC8, *"Modulates receptor-mediated effects"* is considered 'Limited' with some active endpoints in animal *in vivo* studies for AR and induction of P450, however data was largely negative or there was inconsistent activity across similar study designs within specific receptor categories (Fig. 4).

KCC9 "Causes immortalization" (Appendix A. Supplementary data, Table S10). A reliable study was available for evaluating endpoints related to MTBE's ability to cause immortalization (Iavicoli et al., 2002). This study reported an increased number of transformed foci in the rat-1 cell line following MTBE exposure at concentrations ranging from 0.336-0.672 mM (Iavicoli et al., 2002). In another set of publications, an *in vivo* study in rats and an *in vitro* study in a mouse cell line reported changes in c-myc with exposure to MTBE (Zhou et al., 1999a,b); however, these publications had reporting and methodology deficiencies as documented in Table S10.

Overall, the strength of the evidence for KCC9, "*Causes immortalization*" is 'Limited' based on one immortalization assay showing an increased number of transformed foci in a rat-1 cell line (Fig. 4).

KCC10 "Alters cell proliferation, cell death, and nutrient supply" (Appendix A. Supplementary data, Table S11)

In vitro primary human cells (Table 4a). In human umbilical vein endothelial cells (HUVEC) exposed to MTBE concentrations up to 10 mM for 2, 4-, and 24-hours no change in capillary tube formation, a measure of angiogenesis, was established post exposure, however there was a change in morphology with evidence of a decrease in length and width of the capillary tubes (Table 4a) (Kozlosky et al., 2013).

In vitro non-human mammalian primary cells or cell lines (Table 4a). A number of studies in human cell lines and non-human mammalian primary cells and cell lines reported changes in endpoints designated within KCC10. Across all these assays, however, there was no increase in cell proliferation with exposure to MTBE. One study reported no changes in cell proliferation in the human liver cell line LO2, with exposure to high concentrations of MTBE (200 mM) (Shen et al., 2023). Studies in mammalian primary cells or cell lines showed MTBE inhibited cell

Table 4a

In vitro human and non-human mammalian assays for cell proliferation, cell death, and nutrient supply* *(Documentation of all data provided in Supplemental File C; Table S11)*.

Assay Endpoint	Tissue, cell line	Concentration of MTBE[a,b]	Results	Comments/ Quality Assessment	References
In vitro human					
Angiogenesis; Capillary tube morphology,	Primary Human Umbilical Vein Endothelial Cells (HUVEC)	0, 1.25, 2.5, 5.0, and 10.0 mM [1,250–10,000 μM]	↓	No change in capillary tube formation but decrease in length and width; no cytotoxicity measured. **Klimisch = 3**	Kozlosky et al., 2013
In vitro non– human mammalian					
Cell Proliferation; MTT	Rat-1, Fibroblast	0, **0.084, 0.84** mM [84–840 μM]	↓	IC50 = 0.84 mM, so results reported only for lower dose	Iavicoli et al., 2002
Cell Proliferation; MTT	Rat-1, Fibroblast	0, **0.84** mM [840 μM]	↓	Cytotoxicity not observed.	Sgambato et al., 2009
Cell Proliferation; Cell Number	3 T3-1	0.01, 10, 100, 1000 μmol/L (μM)	–	Cytotoxicity measured and observed at > 50,000 μM	Tang et al., 2019
Cell Proliferation; Increased S phase	Rat-1, Fibroblast	0, **0.084, 0.84** mM [84–840 μM]	↑	IC50 was at 0.84 mM, so results reported only for lower dose	Iavicoli et al., 2002
Cell Cycle Arrest; cyclin D1 protein levels	Rat-1, Fibroblast	0, **0.84** mM [840 μM]	↓	Cytotoxicity not observed.	Sgambato et al., 2009
Cell Cycle Arrest; G2/M phase	Rat-1, Fibroblast	0, **0.84** mM [840 μM]	↓	Cytotoxicity not observed.	Sgambato et al., 2009
Cell Cycle Arrest; G1 and G2 arrest	Rat-1, Fibroblast	0, **0.084, 0.84** mM [84–840 μM]	↓	IC50 was at 0.84 mM, so only results reported for lower dose	Iavicoli et al., 2002
Cell Cycle Arrest; G2/M	NIH 3 T3	1, 2, 4 μl/ml [8.39 –33.6 μM]	↑	Cytotoxicity measured; no cytotoxicity observed	Zhou et al., 2000b
Cell Cycle Arrest; G2/M	Primary Rat Immature Leydig Cells	0, 100, **200, 300** mM [100,000–300,000 μM]	↑	No measure of cytotoxicity reported. **Klimisch = 3**	Zhu et al., 2022
Apoptosis; MMP	Primary Rabbit Tracheal Epithelial Cels	0, 0.5, **50, 5000** ppm [5.6–56,721 μM]	–	No effect since cytotoxicity (LDH, cell leakage) measured at 50 ppm and greater.	Wang et al., 2008
Apoptosis; MMP	Primary Liver Mitochondrial Suspensions	0, 10, **50, 250** μM	↓	No measure of cytotoxicity reported. **Klimisch = 3**	Saeedi et al., 2017
Necrosis; Necrotic cells	Primary Spermatogenic Cells	0, 100 ppb, 10, 1000, **3000** ppm [1.13–34,000 μM]	–	Since decreased cell viability at 3000 ppm, only effects earlier were considered	Li et al., 2006
Necrosis; Propidium Iodide	Primary Rabbit Tracheal Epithelial Cells	0, 0.5, 50, **5000** ppm [5.6–56,721 μM]	–	No effect due to cytotoxicity (LDH, cell leakage) measured at 50 ppm and greater.	Wang et al., 2008
Angiogenesis; capillary tube formation and tube length	Primary Rat Brain Endothelial Cells	0, **0.34, 3.4, 34** mM [340–34,000 μM]	↓	No measure of cytotoxicity reported. **Klimisch = 3**	Kozlosky et al., 2013

*All assays with Klimisch scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.

Abbreviations:

NC, no comment.

↑, increased effect.

↓, decreased effect.

–, no activity.

[a] Doses/concentrations with significant effects are bolded.

[b] Concentrations in brackets were calculated as mg/kg.

proliferation at concentrations up to 34 mM (Kozlosky et al., 2013; Iavicoli et al. 2002; Sgambato et al., 2009), with two studies showing no effect on cell proliferation (Tang et al., 2019; Shen et al., 2023). Although no increase in apoptosis/cell death occurred at non-cytotoxic MTBE concentrations, apoptosis increased at concentrations associated with significant cytotoxicity, rendering these findings not reliable for integration of this mechanistic data (Wang et al., 2008; Li et al., 2006). As changes in cell proliferation are inherently related to cell-cycle control alterations, MTBE's effect on these endpoints were also evaluated. One study conducted in NIH/3T3 cells exhibited cell cycle alterations, characterized by a reduction in the population of cells undergoing S phase and an elevation in the proportion of cells in the G2 and M phases. Effects in this study persisted for 48 h, but returned to normal by 96 h post exposure (Zhou et al., 2000b). Two additional studies reported a reduction of cells in G2/M or G1phase arrest (Iavicoli et al., 2002; Sgambato et al., 2009).

In vivo mammalian studies (Table 4b). In a number of reliable studies measures of cell proliferation and cell death were evaluated in MTBE exposed experimental animal models either via inhalation exposure or oral administration. Four of these reliable studies reported increased cell proliferation in female mouse liver and male rat kidney (Prescott-Mathews et al., 1997; Bird et al., 1997; Moser et al., 1996a;1996b). Effects were observed following inhalation exposure of male rats to 3000 or 8000 ppm MTBE, female mice to 8,000 ppm MTBE, or female mice administered MTBE by oral gavage up to a dose level of 1,800 mg/kg-bw/day. No increase in cell proliferation was observed in female rat kidney, male and female mouse liver or male rat testis (Leydig cells) (Zhu et al., 2022), as well as in female mouse uterus, ovary, cervix, pituitary or adrenal glands (Bermudez et al., 2012; Bird et al., 1997; Moser et al., 1996b; Moser et al., 1998). Other signs of increase cellular proliferation included hyperplasia of male rat submandibular lymph nodes following inhalation exposure of male and female rats at 8000 ppm (Lington et al., 1997) and hyperplasia of male rat trachea/lung following inhalation exposure of 60 μL MTBE for 3 mins/day for up to 12 months (Sarhan et al., 2019).

Increased cell death in rat liver and testis (Leydig cells) when measured by mitochondrial membrane potential or protein markers of apoptosis was reported in two reliable studies (Saeedi et al., 2017a; Zhu et al., 2022). Rats were administered up to 1,200 mg/kg-bw/day MTBE by oral gavage (Zhu et al., 2022) or exposed to up to 1,000 μg/L MTBE in a DW study (Saeedi et al., 2017b). Microscopic changes indicative of necrosis was evaluated in two studies that showed an increase in rat kidney proximal tubule necrosis Prescott-Mathews et al., 1997; Robinson et al., 1990). These histological kidney changes were associated with α2u-globulin accumulation, a key event in a male rat specific cancer mechanism (described in detail in sectionon *Mechanisms of tumors in animal models*).

Overall, the strength of the evidence for KCC10, *'Alters cell*

Table 4b
In vivo mammalian cell proliferation, angiogenesis, cell cycle control, necrosis* *(Documentation of all data provided in Supplemental File C; Table S11).*

Assay Endpoint	Species, tissue	MTBE Exposure Concentration or Dose[a,b]	Dose Regimen (route) and Duration of Exposure	Results	Comments/Quality Assessment	Reference
Apoptosis; MMP	SD Rat, Liver	0, 40, **200, 1000** ug/L [0.04–1 mg/kg]	Drinking Water/ Diet; 90 days	↑	NC	Saeedi et al., 2017
Apoptosis; TUNEL	SD Rat, Leydig Cells	0, 300, **600, 1200** mg/kg	Oral Gavage; 21 days	↑	NC	Zhu et al., 2022
Necrosis-Histopathology	Male F-344 Rat, Kidney	0, 400, **1500**, **3000** ppm [400–3000 mg/kg]	Inhalation; 10 days	↑	No effect on terminal body weight	Prescott-Mathews et al., 1997
Cell Proliferation; BrDU	Male F-344 Rat, Kidney	0, 400, **1500, 3000** ppm [400–3000 mg/kg]	Inhalation; 10 days	↑	No effect on terminal body weight	Prescott-Mathews et al., 1997
Cell Proliferation; BrdU	F-344 Rat, Kidney, Liver	0, 400, **3000** and **8000** ppm [400–8000 mg/kg]	Inhalation; 6 hr/ day, 5 days/week for 4 weeks	↑	Increased labeling index (BrdU) in proximal convoluted tubules in the kidneys of male rats, but not female rats at 3000 and 8000 ppm in kidney and at 8000 ppm in liver	Bird et al., 1997
Cell Proliferation; BrDU	Wistar Rat, Kidney	0, 0.5, 3, **15** mg/mL [500–15,000 mg/kg]	Drinking Water; 1,4, 13 weeks	↑	Increased cell proliferation observed at 1 and 4 weeks, but not at 13 weeks	Bermudez et al., 2012
Cell Proliferation; BrDU	B6C3F1 Mouse, Liver	0, **8000** ppm [8000 mg/kg]	Inhalation; 3, 21 days, 16 weeks	↑↓	Increased BrdU labeling at 3 days and decreased at 21 days, no effect at 16 weeks	Moser et al., 1996a
Cell Proliferation; BrDU	CD1Mouse, Liver	0, **8000** ppm [8000 mg/kg]	Inhalation; 3, 21 days	↑	Increased BrdU labeling at 3 days and decreased at 21 days	Moser et al., 1996a
Cell Proliferation; BrDU	B6C3F1 Mouse, Liver	0, **1800** mg/kg bw/ day	Oral Gavage; 3 days	↑	NC	Moser et al., 1996b
Cell Proliferation; BrDU	B6C3F1 Mouse, Liver	0, **7814** ppm [7814 mg/kg]	Inhalation; 6 hr/ day, 5 days/week for 3 or 21 days	↓	Significant decrease in cell proliferation at 21 days, but not 3 days.	Moser et al., 1996b
Cell Proliferation; BrDU and PCNA	B6C3F1Mouse, Uterus	0, **8000** ppm [8000 mg/kg]	Inhalation; 4, 8 months	↓	NC	Moser et al., 1998
Cell Proliferation; BrDU and PCNA	B6C3F1Mouse, Cervix and Vagina, Ovary, Pituitary, Adrenal gland	0, **8000** ppm [8000 mg/kg]	Inhalation; 4, 8 months	–	NC	Moser et al., 1998
Cell Proliferation; Histopathology	Fischer 344 CDF Rat, lymph node	0, 800, 4000, **8000 ppm** [800–8000 mg/kg]	Inhalation; 6 h/ day 5 days per week for 13 weeks	↑	Lymphoid hyperplasia was reported in submandibular lymph node.	Lington et al., 1997
Cell Proliferation; PCNA or PDF gene expression	SD Rat, Leydig Cells	0, 300, 600, 1200 mg/kg	Oral Gavage; 21 days	–	Significant reduction in bodyweight at 1200 mg/kg	Zhu et al., 2022
Cell Cycle Arrest; Cyclin d1 (Ccnd1) gene expression	SD Rat, Leydig Cells	0, 300, 600, **1200** mg/kg	Oral Gavage; 21 days	↓	Significant reduction in bodyweight at 1200 mg/kg	Zhu et al., 2022
Necrosis; Necrotic cells	Wistar Albino Rat, Tracheal Lumen	0, **60** μL/day	Inhalation, 3 mins/day for 6, 12 months	↑	NC	Sarhan, 2022
Cell Proliferation, Histopathology	Wistar Albino Rat, Lung	0, **60** μL/day	Inhalation, 3 mins/day for 6, 12 months	↑	Hyperplasia of lymph nodes in peribronchiolar and perivascular connective tissue, interalveolar septa of air alveoli, and perivascular connective tissue; hyperplasia and degenerative epithelium of mucosal layer.	Sarhan, 2022
Apoptosis; MMP	NMRI Mouse, Liver and Brain	0, **500, 1000** mg/kg bw/day	IP injection; 1 day	↓	Poor reporting on essential study details: sample size for some endpoints, duration of exposure, route of exposure. No systemic toxicity measured.	Faizi, 2020
Necrosis; Histology	New Zealand Rabbit, Gallbladder	NR (only volume reported: 0.5–0.7 mL)	IP injection; 6 h	↑	Total necrosis observed in two out of six animals. Poor reporting and study design: small n (2 control, 6 experimental); limited methods; dose not reported. No systemic toxicity measured.	Mas, 1997

*All assays with Klimisch scores of 1 or 2 are included. Assays with Klimisch scores of 3 are added if activity is indicated but are not used in the evaluation of KCC strength.
Abbreviations:
NC, no comment.
↑, increased effect.
↓, decreased effect.
–, no activity.
NR, not reported.
MMP, mitochondrial membrane potential.
[a] Doses/concentrations with significant effects are bolded.
[b] Concentrations in brackets were calculated as mg/kg.

Table 5
Kidney tumors in male rats through a mechanism the does not operate in humans.

Criteria According to IARC (citation monograph 147), 1999 and EPA, 1991*	Studies that Provide Supporting Data
*Lack of genotoxic activity (agent and or metabolite) based on an overall evaluation of *in vitro* and *in vivo* data (KCC2).	MTBE is not considered a genotoxic agent based on the data summarized in Section 3.2.2.2 (KCC2), which agrees with multiple reviews (McGregor et al., 2006; Bogen and Heilman, 2015; Bus et al., 2022) and also a recent regulatory assessment by ATSDR, 2023. TBA has also been shown to lack genotoxic activity, which was confirmed in a recent publication of an *in vivo* Comet study (Thompson et al., 2023). Although formaldehyde, a metabolite of MTBE, has potential genotoxicity, MTBE has been shown to be negative for both genotoxic and mutagenic activity following *in vivo* exposure (Table 2c).
*Male rat specificity for nephropathy and renal tumorgenicity	MTBE causes kidney tumors in male, but not female, rats or mice (Bird et al., 1997). There was a low incidence of tumors at the mid- and high- exposure concentrations (3000 and 8000 ppm). Kidney tumors were not identified in rats exposed to MTBE via drinking water, although there were male-rat-specific increases in cell proliferation at 1 and 4 weeks of exposure (Dodd et al., 2013).
*Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory.	Exposure to MTBE via inhalation, oral gavage or in drinking water (Bird et al.,1997; Robinson et al., 1990; Prescott-Mathews et al., 1997, 1999; Bermudez et al., 2012) resulted in microscopic lesions in the kidneys of exposed male rats characterized by epithelial-cell necrosis, protein droplet accumulation and karyomegaly within the proximal tubules.
*Identification of the protein accumulating in tubule cells as α2u-globulin	The protein droplets that accumulated stain immunohistochemically for α2u-globulin in kidney sections from male rats exposed to MTBE compared with unexposed male rats (Robinson et al., 1990; Prescott-Mathews et al., 1997; Bermudez et al., 2012).
Reversible binding of the chemical or metabolite to α2u-globulin	Both MTBE and its metabolite TBA have been shown to reversibly bind to a2u-globulin (Poet and Borghoff, 1997; Prescott et al., 1999; Williams and Borghoff, 2001). In MTBE studies in which the concentration of MTBE was measured in male and female rat kidneys, a higher concentration of MTBE was quantitated in male rat kidneys compared to female rat kidney, which was predicted in a PBPK model for MTBE distribution to the male rat kidney being associated with its binding to α2u-globulin (Leavens and Borghoff, 2009; Borghoff et al., 2010).
Induction of sustained increased cell proliferation in the renal cortex	Increased cell proliferation in male, but not female rats with exposure to MTBE was measured over different durations of exposure (Bird et al.,1997; Robinson et al.,1990; Prescott-Mathews et al., 1997; Bermudez et al., 2012).
Similarities in dose–response relationship of the tumour outcome with the histological end points (protein droplet, α2u-globulin accumulation, cell proliferation).	Dose-response relationship demonstrated with protein droplets, pathological lesions associated with accumulation of protein droplet, increase in α2u-globulin concentration and renal cell proliferation up to the exposure concentration that resulted in a low incidence of renal tumors in male rats with chronic exposure (Prescott-Mathews et al., 1997; Bird et al., 1997).

proliferation, cell death, or nutrient supply' is 'Limited' based on minimal/weak activity reported in primary human or non-human mammalian cells or cell lines, activity reported *in vivo* (increased cell proliferation) in animal models in tissues in which tumors were detected in animal cancer studies (i.e., kidney and liver) by mechanisms of tumorigenesis that are considered not to operate in humans (kidney tumors in male rats; Table 5) or associated with high exposure concentrations and toxicity (liver tumors in female mice at 8000 ppm MTBE; Table 1). No alterations in cell proliferation were reported in tissues that did not have corresponding tumors identified in chronic animal cancer studies.

KCC 11: Emerging carcinogenic mechanisms (Appendix A. Supplementary data, Table S12). The combined evidence from twenty-five studies across epidemiological, *in vivo* animal, and *in vitro* assays did not provide evidence that MTBE elicits changes in body weight or BMI with consistent changes in endpoints such as blood lipids, insulin signaling and resistance, and glucose metabolism. In fifteen of sixteen animal studies, MTBE exposure did not increase body weight in any non-transient or dose-dependent manner (Tang et al. 2019; Robinson et al. 1990; Bermudez et al. 2012; Bird et al. 1997; Dong-mei et al. 2009; Elovaara et al. 2007; Li et al. 2008; Lington et al. 1997; Williams and Borghoff, 2000; Williams et al. 2000; Chun et al. 1992; Dodd et al. 2013; Greenough et al. 1980; Guo et al. 2023; de Peyster et al. 2003). An increase in body weight was observed in one study of male Wistar rats at 1 and 100 mg/kg/day (middle and high doses used) (Wang et al. 2025); Further, no increase in body weight was observed in a study of similar design at the same doses (Guo et al. 2023). Several epidemiological studies provide limited and equivocal evidence that MTBE exposure may be associated with increased BMI and insulin resistance (Antonucci et al. 2021; Silva et al. 2019; Guo et al., 2024a,b). These studies, however, also had significant study design and methodological shortcomings (e.g., potential for confounding; cross sectional; limited generalizability), thereby limiting the reliability of these data for integration (Appendix A. Supplementary data, Table S12).

In rodents, MTBE exposure led to inconsistent changes in serum and blood lipid levels, not all of which would be considered adverse (Tang et al. 2019; Robinson et al., 1990; de Peyster et al. 2003; Dong-mei et al. 2009; Saeedi et al., 2017b; Greenough et al. 1980; Guo et al. 2023; Elovaara et al. 2007; Wang et al. 2025), as well as in measures of blood glucose levels (Saeedi et al., 2017b; Robinson et al., 1990; Bermudez et al. 2012; Lington et al. 1997; Greenough et al. 1980; Tang et al. 2019). The few *in vitro* studies available provide limited /equivocal evidence regarding MTBE's effects on adipogenesis, glucose metabolism, and cholesterol efflux (Ren et al. 2021; Tang et al. 2019; Guo et al. 2023; Wang et al. 2025).

The lack of consistent changes in endpoints across *in vivo* studies, along with *in vitro* assay data, does not support that MTBE exposure perturbs pathways associated with insulin resistance and glucose regulation associated with obesity and diabetes.

Other mechanistic evidence

HT in vitro assay data. High throughput (HT) *in vitro* assay data for MTBE (up to 100 μM) available through the ToxCast database and the KC-Hits software tool (i.e., US EPA ToxCast data in vitrodbv_4.0, iarc-imo / kc-hits · GitLab, v0.6.0) report that within a total of 218 assays tested, all were considered "inactive". MTBE's inactivity in these assays is most likely due to MTBE's volatility from testing under the conditions of this HT platform (i.e., unsealed wells). As such, these data are not considered to provide any relevant information to this assessment.

Mechanisms of tumors in animal models. Mechanisms of tumor development in animal models that do not operate in humans have been documented by both EPA (1991), and IARC (Capen et al., 1999, as cited in IARC Preamble), as well as in peer-reviewed literature (e.g., Corton et al., 2018). When IARC first evaluated MTBE in 1999, the case was

made that the kidney tumors in MTBE exposed male rats occurred through a mechanism that does not operate to humans (IARC 1999).

A low but significant increase of liver, kidney, and brain tumors in mice and rats occurred at the high exposure concentration in chronic MTBE studies. The liver tumors may develop through a mechanism associated with PPARα induction that does not operate in humans as described by Corton et al. (2018), however specific data is not available to adequately document this mechanism aside from the fact that MTBE is not genotoxic, induces phase I enzymes in the liver, and results in a mitogenic cell proliferative response (data supported in KCC2, KCC8, KCC10). Investigation of Leydig cell tumor formation in rats is almost exclusively associated with elevated levels of luteinizing hormone (LH) (Clegg et al., 1997; Cook et al., 1999), a change in LH has not been demonstrated in rats administered MTBE (De Peyster et al., 2003). MTBE inhalation exposure or oral administration resulted in increased Leydig cell tumor incidence in F344 and Sprague Dawley rats, respectively, following chronic exposure (Belpoggi et al., 1995;1997; 1998; Bird et al., 1997). Sprague-Dawley rats typically have a low spontaneous incidence of Leydig cell tumors (3–4 %) compared with 90–100 % in F344 rats (Maronpot et al., 2016). MTBE also produced a statistically significant increase in Leydig cell tumor incidence in rats dosed with 1000 mg/kg-bw of MTBE (34.3 versus 7.7 % for the control). In this study, MTBE administration was stopped after 104 weeks, and the animals were maintained until death, with male rats in the high dose group living longer than controls. This study was also reported to have limitations and designated as not reliable.

Strong evidence exists, however, that MTBE causes α2u-globulin nephropathy, a mechanism that results in a low incidence of renal tumors in male, but not female rats, since female rats do not synthesize α2u-globulin in the liver. The ability of MTBE to induce protein droplet accumulation, due to the accumulation of α2u-globulin caused by the binding of MTBE to this protein decreasing its rate of catabolism (Poet and Borghoff, 1997; Prescott et al., 1999; Williams and Borghoff, 2001; Leavens and Borghoff, 2009; Borghoff et al., 2010) and resulting in cell death and an increase in renal cell proliferation, in male but not female Fischer 344 rats exposed to MTBE (up to 3013 ppm) for 6 h a day for 10 consecutive days. Microscopic lesions in the kidneys of exposed male rats were characterized by epithelial-cell necrosis, protein droplet accumulation and karyomegaly within the proximal tubules. In addition, occasional epithelial cell exfoliation into the tubular lumen was observed. The protein droplets that accumulated stained immunohistochemically for α2u-globulin in kidney sections from male rats exposed to MTBE, with a statistically significant increase in the concentration of α2u-globulin in male rats exposed to 3013 ppm MTBE when compared with unexposed males. A strong positive correlation ($r = 0.994$) was demonstrated between an increase in labeling index of cells in the renal cortex (cell proliferation) and mean α2u-globulin concentration. The results of this study indicate that MTBE is a mild inducer of α2u-globulin nephropathy and enhanced renal cell proliferation in male, but not female, rats (Prescott-Mathews et al., 1997) that is associated with a low increase of kidney tumors following chronic exposure. As such, this study, together with supporting publications, demonstrate that MTBE causes kidney tumors in male rats via a α2u-globulin mechanism which does not operate in humans (Table 5) according to criteria identified by EPA and IARC (Capen et al., 1999; EPA, 1991).

Evidence integration

Overall, there were no reliable human data, two standard 2-year cancer studies in rats (inhalation and drinking water) and one in mice (inhalation), that reported an increased incidence of kidney and brain tumors in male rats, and liver tumors in female mice. Based on the low incidence of three tumor types (liver; kidney; brain) at high exposure concentrations across three standard cancer bioassays, in concert with the lack of genotoxicity and overall strength of the mechanistic data being limited, MTBE is unlikely to be a carcinogenic hazard in humans based on integration of evidence streams across animal cancer studies and mechanistic data (Fig. 5).

Discussion

In this systematic review of MTBE's evidence base associated with

Animal Cancer Studies	Mechanistic Data (Genotoxicity and Other Mechanistic Data)	Human Cancer Study
Three reliable standard cancer studies. Two non-standard cancer studies, one of which was unreliable; neither provided support for findings in standard cancer studies. Tumors identified: • Liver hepatocellular adenomas (low incidence at highest exposure concentration) in female mice via inhalation exposure. • Brain tumors (low incidence at high exposure concentration) in male rats via drinking water. • Kidney tumors (low incidence at high exposure concentrations) in male rats via inhalation exposure through mechanism that does not operate in humans (see Table 5).	Overall evidence in relevant models indicates that MTBE is not mutagenic or genotoxic. No mechanistic data available in exposed humans. General lack of activity in a few assays using primary human cells or tissues. Limited or inconsistent activity in mechanistic animal studies *in vivo*, human cell lines, and mammalian primary cells or cell lines. Most mechanistic data that showed activity were not consistent across similar study types and biological levels of complexity. Cell proliferative activity was high and associated with cytotoxic and/or mitogenic mechanisms of kidney and liver tumors, respectively; these mechanisms are not likely to operate in humans.	One human epidemiological study considered unreliable based on study design and lack of clarity in the relationship between exposure to MTBE and tumor outcome.
"Limited" in Animal Models	**"Limited" Mechanistic Data**	**"Inadequate" in Humans**
Integrated conclusion: **MTBE is unlikely to be a carcinogenic hazard in humans based on integration of evidence streams across animal cancer studies and mechanistic data.**		

Fig. 5. Integration of the totality of data informing the potential carcinogenic hazard of MTBE in humans.

carcinogenic activity, one human study, five experimental animal cancer studies, along with a significant volume of mechanistic data mapped across the KCCs was reviewed to assess the potential of MTBE's carcinogenic hazard in humans. The sole human cancer epidemiological study identified had significant limitations for evaluating the association between MTBE exposure and pancreatic cancer in the population evaluated (Rodriguez et al., 2024). Consequently, the overall assessment was based on the standard cancer bioassays in animal models and significant mechanistic evidence considered reliable. No mechanistic data were available in exposed humans, and only limited data were available from human primary cells or tissues. Most of the mechanistic endpoints were derived from animal *in vivo* models and non-human mammalian primary cells and cell lines, providing data across the KCCs. Following data integration, the conclusion reached is that MTBE is unlikely to be a carcinogenic hazard in humans.

In a thorough review of MTBE and its cancer potency, Bogen and Heilman (2015), outlined agency cancer classifications for MTBE. There was overall agreement that MTBE is an animal carcinogen at high exposure concentrations, however it is not genotoxic with no evidence to support a concern for MTBE as a potential human carcinogen (EPA, 1997; IPCS, 1998; IARC, 1999; California EPA, 1999). This was recently summarized in the ATSDR MTBE Toxicology Profile (2023). In the current evaluation where all the animal cancer bioassay data are reviewed including the drinking water study published by Dodd et al. (2013) along with significant mechanistic data across the ten KCCs, the overall conclusion remains the same.

Three standard animal cancer studies reported a low incidence of tumors that developed following chronic MTBE exposure (Bird et al., 1997; Dodd et al., 2013). Female mice exposed to 8,000 ppm of MTBE via inhalation developed hepatocellular adenomas, while male rats exposed to 3,000 ppm developed kidney tumors. Additionally, male rats exposed to MTBE via drinking water (7.4 mg/mL) developed brain tumors. Testicular interstitial cell tumors also were observed to increase in male rats exposed to 8,000 ppm MTBE, but as noted by Steinbach et al. (2015), these tumors are a common age-related finding in rats, with the incidence approaching 100 % in rats at the end of their lifespan. Although exposure to 8,000 ppm MTBE (the highest exposure concentration) resulted in a statistically significant increase in these tumors in male rats, this result was likely attributable to the incidence of these tumors in the control group being on the low end of the historical range for this tumor type (Bird et al., 1997). As noted by Maronpot et al., (2016) the historical control rate of testicular tumors in F344 rats is reported to have ranged from 54% to as high as 98% within studies conducted in F344 by the NTP. Most studies reporting a treatment-related Leydig cell tumor response were inhalation exposures where the spontaneous incidence of these tumors in control animals was lower compared to the incidence following other routes of test article administration. Together this information supports that in studies with a high background incidence of a tumor type there is insufficient statistical power to detect effects. As such, these tumors were not considered relevant for assessing human carcinogenic hazard in this evaluation or in previous evaluations of MTBE (IARC 1999, Bogen and Heilman, 2015; ATSDR, 2023).

Regarding the findings in the male rat kidney, only one cancer study showed a statistically significant increase in renal adenoma and carcinoma incidence in male rats when exposed via inhalation to 3,000 ppm MTBE (calculated dose: 310 mg/kg-bw/day 5 days per week) (Bird et al., 1997). This study was terminated early because of significant mortality of male rats in the 3,000 and 8,000 ppm exposure groups. The primary mortality cause was associated with the increased incidence and severity of chronic progressive nephropathy (CPN), a common age-related lesion specific to rats that can be exacerbated by exposure to some chemicals (Hard et al., 2013). Although unusual, an increase in kidney tumors in rats has been associated with high-grade CPN particularly in male rats, but occasionally in female rats (Hard et al., 2012). The severity of CPN differs with rat strain and rodent diet which may explain the differences in male rat specific tumor outcomes in these different cancer bioassays. In the MTBE drinking water cancer study conducted in Wistar HAN rats, MTBE exposure at the highest tested concentration (7.5 mg/mL, equivalent to a calculated dose: 333 mg/kg-bw/day) resulted in an increased incidence of CPN (Dodd et al., 2013) without resulting in kidney tumors. Although the calculated MTBE dose level was comparable to the inhalation study, no increased mortality or neoplastic renal lesions were observed in male rats. The lack of kidney tumors in male rats exposed to MTBE via drinking water—with what appears to be a comparable dose level to the rat inhalation study (Bird et al., 1997)—is most likely due to toxicokinetic differences following this route of exposure, variability in strain-specific responses, and, perhaps, differences in rodent diets used in these studies. Notably, high protein diets that can influence age-related findings in rodents were changed to low protein diets during this period between the conduct of the Bird et al., 1997 and the Dodd et al., 2013 studies (Rao, 1997). The comparison between these two studies emphasizes the lack of reproducibility in tumor outcomes associated with nongenotoxic chemicals like MTBE that cause a low tumor incidence (i.e., liver, kidney, brain). Also, the kidney tumors that developed following MTBE inhalation exposure have been shown to operate through a mechanism not relevant in humans (Borghoff et al. 1990; Swenberg and Lehman-McKeeman, 1999; Hard et al., 1993; Goyak et al., 2022) when considering criteria by either EPA or IARC that are used to demonstrate if a chemical operates through the α2u-globulin nephropathy mechanism resulting in male rat specific kidney tumors (EPA, 1991; Capen et al., 1999). Data from the literature supports that MTBE binds to the male rat protein α2u-globulin, leading to increased cell necrosis, cell proliferation, and associated kidney pathology. This mechanism does not operate in humans since humans do not synthesize α2u-globulin, as supported by that fact that this protein was not identified in human kidneys (Borghoff and Lagarde, 1993). Consequently, the kidney tumor response in rats is not considered in this evaluation of MTBE as a cancer hazard to humans.

Other tumor responses observed included hepatocellular adenomas in female CD-1 mice and brain astrocytomas in male rats (Bird et al., 1997; Dodd et al., 2013). The increased hepatocellular adenoma incidence (20%) was reported following chronic MTBE inhalation exposure (8,000 ppm) and represented an increase over the historical control range for this tumor in CD-1 mice (0–4%). Authoritative entities did not place concern on this response, driven by exposure to high MTBE concentrations that exceeded the MTD in this study (ATSDR, 2023). Brain astrocytomas tumors were identified in a chronic MTBE drinking water study conducted in male Wistar rats (Dodd et al., 2012). A statistically increased astrocytoma incidence was reported at 7.5 mg/mL MTBE (calculated dose: ~333 mg/kg-bw/day) when assessed using a statistical trend test (Cochran), but not when using a pairwise comparison (Fisher's exact test $p < 0.181$). Spontaneous tumors occurring in the central nervous system in rats is typically very low (<1%) (Nagatani et al., 2013; Rice and Wilbourn, 2000; Sills et al., 1999), as such the consideration of historical tumor incidence can be very useful for understanding the significance of this finding. Unfortunately, historical control data were not available for this cancer study (Dodd et al., 2013) based the lack of prior use of these Wistar rats in the testing laboratory. The marginal statistical significance for these brain tumors in the high dose group may suggest that these tumors occurred by chance, especially since brain tumors did not occur in the F344 rats exposed to MTBE via inhalation (Bird et al., 1997), or in the nonstandard study conducted in Sprague Dawley rats via oral gavage (Belpoggi et al., 1995;1997). One way to considered if this finding of brain tumors is a false positive is by adjusting the level of statistical significance based on the rarity of the tumor type. Haseman (1983) developed a statistical procedure requiring a $P = 0.05$ for pairwise comparisons for rare tumors and $P = 0.01$ for common tumors with a frequency greater than 1 %. This procedure was based on the idea that the higher number of tumors present, the greater the likelihood of a false positive result. This approach was subsequently extended to the use of linear trend tests, in which p values of $P < 0.005$

and $P < 0.025$ were considered appropriate cut-off values for common and rare tumors, respectively (Lin and Rahman, 1998). When using this approach, the false positive rate was about 10%, and, as such, the brain astrocytomas observed following MTBE exposure would not be considered significant. Dodd et al. did not perform immunohistochemical analysis or provide histological description of the brain tumors identified, however, reclassification of astrocytomas, often to malignant microglial tumors, has been described by Kolanda-Roberts et al. (2013) when immunohistochemical stains are applied. As such, the brain tumors found in these rats are likely not to be relevant to humans since the formation of glial tumors has been associated with the presence of Epstein-Barr virus in humans (Akhtar et al., 2018). When considering the totality of the evidence, astrocytomas were not identified in mice chronically exposed to MTBE, nor consistently found across three rat studies, despite the use of higher MTBE doses (~333 mg/kg-bw/day versus 1,000 mg/kg-bw/day, 4 days/week in the Belpoggi study) (Bird et al., 1997; Dodd et al., 2013; Belpoggi et al., 1995;1997). With a non-genotoxic chemical like MTBE, where exposure results in a low incidence of tumors responses (female mouse liver, male rat kidney, and male rat brain) across three separate studies, the lack of a reproducible tumor response is often driven by differences in species or strain sensitivity, route of exposure, dose levels, and or rodent diet and not necessarily predictive of carcinogenic activity in humans.

When considering both animal cancer and the mechanistic data, keeping in mind that MTBE is metabolized into equimolar amounts of TBA and formaldehyde in both humans and rats becomes especially important. The role of these metabolites in cancer outcomes have been previously considered (IARC, 1999; Health Canada, 2006; Bus et al., 2022; ATSDR, 2023). TBA is considered non-genotoxic (McGregor 2010; Thompson et al., 2024) and induces a minimal or low incidence of kidney tumors in male rats and thyroid tumors in mice, both of which are proposed to occur through mechanisms not relevant to humans (Borghoff et al., 2001; McGregor et al., 2005; Blanck et al., 2010). In evaluating MTBE's mechanistic data, studies investigating KCC1 indicate that, under specific conditions, MTBE's metabolism to formaldehyde may lead to DNA adducts and/or DNA-protein crosslinks. In animal *in vivo* studies, since these studies did not differentiate whether DNA-adducts were formed vs. ^{14}C derived from radiolabeled MTBE was incorporated into the one-carbon pool, these studies were not considered reliable based on methodology. In the *in vitro* rodent hepatocyte experiments reported by Casanova and Heck (1997), a low level of DNA-protein crosslinks/ RNA-formaldehyde adducts were formed but were not dose-dependent and did not change when metabolism was induced. The volatility of MTBE was controlled in these experiments. Overall, this supported that the production of formaldehyde from the metabolism of MTBE was slow relative to the rate of formaldehyde metabolism, indication its formation does not appear to be involved in any of the tumor responses, supported by the lack mutagenic and/or genotoxic outcomes in animal *in vivo* studies. Additionally, no evidence exists in humans (*in vivo* or *in vitro* models) to support formation of DNA adducts or DNA-protein crosslinks. As such, no evidence exists that formaldehyde plays a role in MTBE's carcinogenic outcome in animal models. Studies investigating high MTBE exposure concentrations in rats that examine KCC2 report no mutagenic or genotoxic activity (See Tables 2a-c). These results support the finding that the formaldehyde formed is detoxified to acetone and subsequently incorporated in the 1-carbon pool.

At the outset of this evaluation, there appeared to be a significant mechanistic evidence base to evaluate MTBE's carcinogenic potential across different models. However, the majority of the mechanistic data available for MTBE was found to be in non-human mammalian primary cells or cell lines and experimental animal *in vivo* models. No measures of activity were made in exposed humans, and limited measures of activity were investigated in human primary cells and tissues. Also, a major concern with evaluating MTBE *in vitro* is its volatility, which make it difficult for the concentration of MTBE to be maintained throughout the duration of the assay. This challenge can be controlled using an incubation vessel with limited surface area (size or type), and/or by sealing the headspace when feasible. These controls are not easy to administer in cell-based assays, however. Although several *in vitro* assays did control for MTBE's volatility (i.e. Casanova and Heck, 1997; Poet and Borghoff, 1997, etc.), most studies did not specify whether MTBE volatility was controlled, nor was the concentration in the test assay measured. In HT assays reported in the EPA CompTox database, assay plates are not sealed, and all MTBE HT assay data were found to be inactive (EPA, 2024a). This result is attributed to MTBE's volatility with an open (non-sealed) platform, potentially yielding false negative data. Many *in vitro* studies in this assessment (See Appendix A. Supplementary data. Table S1) used extremely high MTBE concentrations (in the mM range), however the final concentration was still never confirmed, and, in many cases, cytotoxicity not evaluated. As a result, the reliability of most of the *in vitro* data, whether negative or positive, is questionable.

Given concern for the overall relevance of the *in vitro* assay data based on MTBE's volatility, the weight of the mechanistic data evaluation relied heavily on *in vivo* animal studies in which the MTBE dose/concentration could be controlled. As such, the studies providing the most reliable data were for KCC2 (genotoxicity) and KCC10 (alters cell proliferation, cell death, or nutrient supply) were animal *in vivo* studies. The metabolism of MTBE results in the same metabolites in exposed humans and rats which emphasizes the ability of these animal studies to provide mechanistic insight into MTBE's potential carcinogenicity in humans. Overall, the mutagenic or genotoxic activity of MTBE in animal *in vivo* models were negative, confirming a lack of concern for this activity occurring in exposed humans. The animal *in vivo* data also showed consistency in cell necrosis and cell proliferation indicating that exposure to high concentrations of MTBE drive high dose cytotoxic key events for development of mouse liver tumors and male rat specific renal tumors.

Conclusion

Based on the integration of reliable studies and endpoint methodology, three standard experimental animal studies, along with mechanistic data organized, evaluated, and integration across ten KCCs was considered to inform the human carcinogenic potential of MTBE (Fig. 5). Although a low, and non-reproducible tumor incidence was identified in three standard reliable cancer studies, MTBE's lack of genotoxic activity and with limited strength of activity across the other KCCs does not support concern for carcinogenic activity in humans. As such, a biologically plausible mechanism leading to carcinogenic activity of MTBE that would operate in humans is not evident within this significant evidence base supported mainly by animal *in vivo* studies.

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CRediT authorship contribution statement

S.J. Borghoff: Conceptualization, Formal analysis, Investigation, Funding acquisition, Writing – original draft, Writing – review & editing. **B.N. Rivera:** Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **S. Fitch:** Methodology, Data curation, Writing – review & editing. **A.N. Buerger:** Formal analysis, Data curation, Writing – original draft. **N.Y. Choksi:** Formal analysis, Data curation, Writing – review & editing. **A. Franzen:** Formal analysis, Data curation, Writing – review & editing. **M.J. Vincent:** Formal analysis, Writing – original draft, Writing – review & editing. **T. Covington:** Formal analysis, Writing – original draft. **J. Bus:** Formal analysis, Writing – review & editing. **E. Rushton:** Conceptualization, Writing – review & editing. **I.A. Lea:** Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100224.

Data availability

All data referenced has been cited.

References

Akhtar, S., Vranic, S., Cyprian, F.S., Al Moustafa, A.E., 2018. Epstein-barr virus in gliomas: cause, association, or artifact? Front. Oncol. 2018 (8), 123. https://doi.org/10.3389/fonc.2018.00123.

Amberg, A., Rosner, E., Dekant, W., 1999. Biotransformation and kinetics of excretion of methyl-tert-butyl ether in rats and humans. Toxicol. Sci. 51 (1), 1–8. https://doi.org/10.1093/toxsci/51.1.1.

Amberg, A., Rosner, E., Dekant, W., 2001. Toxicokinetics of methyl tert-butyl ether and its metabolites in humans after oral exposure. Toxicol. Sci. 61 (1), 62–67. https://doi.org/10.1093/toxsci/61.1.62.

Antonucci, A., Protano, C., Astolfi, M.L., Mattei, V., Santilli, F., Martellucci, S., Vitali, M., 2021. Exposure profile to traffic related pollution in pediatric age: a biomonitoring study. Int. J. Environ. Res. Pub. Health 18 (19), 10118. https://doi.org/10.2290/ijerph181810118.

ARCO Chemical Company, 1980. Acute tox study on meth t-butyl ether & letter from Litton Bionetics to ARCO on evaluation rationale & additional info regarding SCE & chromosome aberration assays with cover letter.

ATSDR, 2023. Toxicological Profile for Methyl Tert-Butyl Ether (MTBE). Atlanta GA: Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Service, Public Health Service. https://www.atsdr.cdc.gov/toxprofiles/tp91.pdf. Accessed October 2024.

Badr, A.A., 2019. Toxic effects of low doses of methyl-tertiary butyl ether on hematological indices in the male rats. Trends Pharm. Sci. 5 (4), 173–176. https://doi.org/10.30476/TIPS.2020.84445.1033.

Belpoggi, F., Soffritti, M., Maltoni, C., 1995. Methyl-tertiary-butyl ether (MTBE) — a gasoline additive — causes testicular and lympho haematopoietic cancers in rats. Toxicol. Ind. Health. 11 (2), 119–149. https://doi.org/10.1177/074823379501100202.

Belpoggi, F., Soffritti, M., Filippini, F., Maltoni, C., 1997. Results of long-term experimental studies on the carcinogenicity of methyl tert-butyl ether. Ann. N.Y. Acad. Sci. 837, 77–95. https://doi.org/10.1111/j.1749-6632.1997.tb56865.x.

Belpoggi, F., Soffritti, M., Maltoni, C., 1998. Pathological characterization of testicular tumours and lymphomas-leukaemias, and of their precursors observed in Sprague-Dawley rats exposed to methyl-tertiary butyl-ether (MTBE). Eur. J. Oncol. 3 (3), 201–206.

Beronius, A., Molander, L., Zilliacus, J., Rudén, C., Hanberg, A., 2018. Testing and refining the Science in Risk Assessment and Policy (SciRAP) web-based platform for evaluating the reliability and relevance of in vivo toxicity studies. J. Appl. Toxicol. 38 (12), 1460–1470. https://doi.org/10.1002/jat.3648.

Bermudez, E., Willson, G., Parkinson, H., Dodd, D., 2012. Toxicity of methyl tertiary-butyl ether (MTBE) following exposure of Wistar Rats for 13 weeks or one year via drinking water. J. Appl. Toxicol. 32, 687–706. https://doi.org/10.1002/jat.1674.

Bernauer, U., Amberg, A., Scheutzow, D., Dekant, W., 1998. Biotransformation of 12C- and 2-13C-labeled methyl tert-butyl ether, ethyl tert-butyl ether, and tert-butyl alcohol in rats: identification of metabolites in urine by 13C nuclear magnetic resonance and gas chromatography/mass spectrometry. Chem. Res. Toxicol. 11 (6), 651–658. https://doi.org/10.1021/tx970215v.

Bird, M.G., Burleigh-Flayer, H.D., Chun, J.S., Douglas, J.F., Kneiss, J.J., Andrews, L.S., 1997. Oncogenicity studies of inhaled methyl tertiary-butyl ether (MTBE) in CD-1 mice and F-344 rats. J. Appl. Toxicol. 17 (Sup 1), S45-S55. Doi: 10.1002/(SICI)1099-1263(199705)17:1+<S45::AID-JAT410>3.0.CO;2-K.

Blanck, O., Fowles, J., Schorsch, F., Pallen, C., Espinasse-Lormeau, H., Schulte-Koerne, E., Totis, M., Banton, M., 2010. Tertiary butyl alcohol in drinking water induces phase I and II liver enzymes with consequent effects on thyroid hormone homeostasis in the B6C3F1 female mouse. J. Appl. Toxicol. 30 (2), 125–132. https://doi.org/10.1002/jat.1478.

Bogen, K.T., Heilman, J.M., 2015. Reassessment of MTBE cancer potency considering modes of action for MTBE and its metabolites. Crit. Rev. Toxicol. 45 (Suppl 1), 1–56. https://doi.org/10.3109/10408444.2015.1052367.

Borghoff, S.J., Short, B.G., Swenberg, J.A., 1990. Biochemical mechanisms and pathobiology of alpha 2u-globulin nephropathy. Annu. Rev. Pharmacol. Toxicol. 30 (April), 349–367. https://doi.org/10.1146/annurev.pa.30.040190.002025.

Borghoff, S.J., Lagarde, W.H., 1993. Assessment of binding of 2,4,4-trimethyl-2-pentanol to low-molecular-weight proteins isolated from kidneys of male rats and humans. Toxicol. Appl. Pharmacol. 19 (2), 228–235. https://doi.org/10.1006/taap.1993.1064.

Borghoff, S.J., Prescott, J.S., Janszen, D.B., Wong, B.A., Everitt, J.I., 2001. Alpha 2u-globulin nephropathy, renal cell proliferation, and dosimetry of inhaled tert-butyl alcohol in male and female F-344 rats. Toxicol. Sci. 61 (1), 176–186. https://doi.org/10.1093/toxsci/61.1.176.

Borghoff, S.J., Parkinson, H., Leavens, T.L., 2010. Physiologically based pharmacokinetic rat model for methyl tertiary-Butyl Ether; Comparison of selected dose metrics following various MTBE exposure scenarios used for toxicity and carcinogenicity evaluation. Toxicol. 275 (1–3), 79–91. https://doi.org/10.1016/j.tox.2010.06.003.

Burleigh-Flayer, H.D., Chun, J.S., Kintigh, W.J., 1992. Methyl Tertiary Butyl Ether: Vapor Inhalation Oncogenicity Study in Cd-1 Mice with Cover Letter dated 10/29/92. Export, PA: Bushy Run Research Center. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/OTS0558685.xhtml.

Bus, J.S., 2017. IARC use of oxidative stress as key mode of action characteristic for facilitating cancer classification: Glyphosate case example illustrating a lack of robustness in interpretative implementation. Regul. Toxicol. Pharmacol. 86 (June), 157–166. https://doi.org/10.1016/j.yrtph.2017.03.004.

Bus, J.S., Gollapudi, B.B., Hard, G.C., 2022. Methyl-tert-butyl ether (MTBE): integration of rat and mouse carcinogenicity data with mode of action and human and rodent bioassay dosimetry and toxicokinetics indicates MTBE is not a plausible human carcinogen. J. Toxicol. Environ. Health b. 25 (4), 135–161. https://doi.org/10.1080/10937404.2022.2041516.

California EPA (California Environmental Protection Agency), 1999. Public Health Goal for Methyl Tertiary Butyl Ether (MTBE) in Drinking Water. March. https://oehha.ca.gov/media/downloads/water/chemicals/phg/mtbef.pdf.

Capen, C.C., Dybing, E., Rice, J.M., Wilbourn, J.D., 1999. Species differences in thyroid, 15 kidney and urinary bladder carcinogenesis. IARC Sci. Pub. 147, 15–32. https://publications.iarc.fr/Book-And-Report-Series/Iarc-Scientific-Publications/Species-Differences-In-Thyroid-Kidney-And-Urinary-Bladder-Carcinogenesis-1999.

Casanova, M., Heck, H., 1997. Lack of evidence for the involvement of formaldehyde in the hepatocarcinogenicity of methyl tertiary-butyl ether in Cd-1 mice. Chem. Biol. Interact. 105 (2), 131–143. https://doi.org/10.1016/S0009-2797(97)00043-4.

Chen, C.S., Hseu, Y.C., Liang, S.H., Kuo, J.Y., Che, S., 2008. Assessment of genotoxicity of methyl-tert-butyl ether, benzene, toluene, ethylbenzene, and xylene to human lymphocytes using comet assay. J. Haz. Mats. 153 (1–2), 351–356. https://doi.org/10.1016/j.jhazmat.2007.08.053.

Chun, J.S., Burleigh-Flayer, H.D., Kintigh, W.J., 1992. Final Report, Methyl Tertiary Butyl Ether - Vapor Inhalation Oncogenicity Study in Fischer 344 Rats, with Cover Letter Dated 11/19/92. Bushy Run Research Center, Export, PA.

Cook, J.C., Klinefelter, G.R., Hardisty, J.F., Sharpe, R.M., Foster, P.M.D., 1999. Rodent Leydig cell tumorigenesis: a review of the physiology, pathology, mechanisms, and

relevance to humans. Crit. Rev. Toxicol. 29 (2), 169–261. https://doi.org/10.1080/10408449991349203.
Corton, J.C., Peters, J.M., Klaunig, J.E., 2018. The PPARα-dependent rodent liver tumor response is not relevant to humans: addressing misconceptions. Arch. Toxicol. 92 (1), 83–119. https://doi.org/10.1007/s00204-017-2094-7.
Clegg, E.D., Cook, J.C., Chapin, R.E., Foster, P.M., Daston, G.P., 1997. Leydig cell hyperplasia and adenoma formation: Mechanisms and relevance to humans. Reprod. Toxicol. 11 (1), 107–121. https://doi.org/10.1016/s0890-6238(96)00203-1.
Darwish, I.A.E., Mosallam, S.A.E., 2019. Chromosome aberrations in bone marrow cells of rats treated with MTBE. Pak. J. Pharm. Sci. 32 (1), 89–93.
de Peyster, A., MacLean, K.J., Stephens, B.A., Ahern, L.D., Westover, C.M., Rozenshteyn, D., 2003. Subchronic studies in Sprague-Dawley rats to investigate mechanisms of MTBE-induced Leydig cell cancer. Toxicol. Sci. 72 (1), 31-42. Doi: 0.1093/toxsci/kfg011.
de Peyster, A., Mihaich, E., Kim, D.H., Elyea, W.A., Nemec, M.J., Hirakawa, B.P., Leggieri, S.E., 2014. Responses of the steroidogenic pathway from exposure to methyl-tert-butyl ether and tert-butanol. Toxicol. 319 (May 7), 23–37. https://doi.org/10.1016/j.tox.2014.01.015.
de Peyster, A., Rodriguez, Y., Shuto, R., Goldberg, B., Gonzales, F., Pu, X., Klaunig, J.E., 2008. Effect of oral methyl-t-butyl ether (MTBE) on the male mouse reproductive tract and oxidative stress in liver. Reprod. Toxicol. 26 (3–4), 246–253. https://doi.org/10.1016/j.reprotox.2008.08.009.
Dodd, D., Willson, G., Parkinson, H., Bermudez, E., 2013. Two-year drinking water carcinogenicity study of methyl tertiary-butyl ether (MTBE) in Wistar rats. J. Appl. Toxicol. 33 (7), 593–606. https://doi.org/10.1002/jat.1776.
Dong-mei, L., Yi, G., Chun-Tao, Y., Yu-feng, H., Xiao-dong, H., 2009. Effects of subchronic methyl tert-butyl ether exposure on male Sprague-Dawley rats. Toxicol Ind Health. 25 (1), 15–23. https://doi.org/10.1177/0748233708101594.
Du, H.F., Xu, L.H., Wang, H.F., Liu, Y.F., Tang, X.Y., Liu, K.X., Peng, S.X., 2005. Formation of MTBE-DNA adducts in mice measured with accelerator mass spectrometry. Environ. Toxicol. 20 (4), 397–401. https://doi.org/10.1002/tox.20124.
EIA (Energy Information Administration), 2020. MTBE, Oxygenates, and Motor Gasoline. https://www.eia.gov/outlooks/steo/special/pdf/mtbe.pdf.
Elmore, S.A., Rehg, J.E., Schoeb, T.R., Everitt, J.I., Bolon, B., 2023. Pathologists' perspective on the study design, analysis, and interpretation of proliferative lesions in lifetime and prenatal rodent carcinogenicity bioassays of aspartame. Food Chem. Toxicol. 171 (Jan), 113504. https://doi.org/10.1016/j.fct.2022.113504.
Elovaara, E., Stockmann-Juvala, H., Mikkola, J., Gelboin, H.V., 2007. Interactive effects of methyl tertiary-butyl ether (MTBE) and tertiary-amyl methyl ether (TAME), ethanol and some drugs: Triglyceridemia, liver toxicity and induction of CYP (2E1, 2B1) and phase II enzymes in female Wistar rats. Environ. Toxicol. Pharmacol. 23 (1), 64–72. https://doi.org/10.1016/j.etap.2006.07.003.
EPA (U.S. Environmental Protection Agency), 1997. Drinking Water Advisory: Consumer Acceptability Advice and Health Effects Analysis on Methyl Tertiary-Butyl Ether (MTBE). September. EPA 822-F-97-008. U.S. Environmental Protection Agency, Office of Research and Development, Washington, D.C. https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=20003HO4.txtDocument Display (PURL) | NSCEP | US EPA.
EPA (U.S. Environmental Protection Agency), 2007. State Actions Banning MTBE (Statewide). August. EPA420-B-07–013. U.S. Environmental Protection Agency, Washington, D.C. https://permanent.fdlp.gov/gpo63501/ZyPDF.pdf.
EPA (U.S. Environmental Protection Agency), 2024a. Methyl tert-butyl ether: Bioactivity – TOXCAST Summary. CompTox Chemicals Dashboard v2.5.0. Accessed: March 2024. https://comptox.epa.gov/dashboard/chemical/invitrodb/DTXSID3020833.
EPA (U.S. Environmental Protection Agency), 2024b. Methyl tert-butyl ether: ToxCast Model Predictions. CompTox Chemicals Dashboard v2.5.0. Accessed: October 2024. https://comptox.epa.gov/dashboard/chemical/bioactivity-toxcast-models/DTXSID3020833.
EPA (U.S. Environmental Protection Agency) 2015. Federal Register/Vol 80, No. 135/Wednesday, July 15, 2015.
EPA (U.S. Environmental Protection Agency), 1991. Alpha 2u -Globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat. September. EPA/625/3– 91/019F. U.S. Environmental Protection Agency, Office of Water, Washington, D.C. https://archive.epa.gov/raf/web/html/alpha2u-globulin.html.
EPA BRP (U.S. Environmental Protection Agency Blue Ribbon Panel), 1999. Achieving Clean Air and Clean Water: The Report of the Blue Ribbon Panel on Oxygenates in Gasoline. Washington, D.C., September 15. https://www.epa.gov/sites/default/files/2014-10/documents/r99021.pdf.
Ghasemi, S., Ahmadi, F., 2014. The study of binding of methyl tert-butyl ether to human telomeric G-quadruplex and calf thymus DNA by gas chromatography, a thermodynamic discussion. J. Chromatog. B. 971 (Nov 15), 112-119. Doi: 10.1016.2014.09011.
Gift, J.S., Caldwell, J.C., Jinot, J., Evans, M.V., Cote, I., Vandenberg, J.J., 2013. Scientific considerations for evaluating cancer bioassays conducted by the Ramazzini Institute. Environ Health Perspect 121, 11–12.
Gollapudi, B.B., Bhaskar, R., 2024. Mutagenicity evaluation of methyl tertiary- butyl ether in multiple tssues of transgenic rats following whole body inhalation exposure. Environ. Mol. Mutagen. 65 (6–7), 222–229. https://doi.org/10.1002/em.22616.
Guo, M., Li, M., Chen, L., Wang, H., Wang, J., Niu, P., Ma, J., 2023. Glutaminase 1 isoform up-regulation associated with lipid metabolism disorder induced by methyl tertiary-butyl ether in male rats. Exotoxicol. Environ. Saf. 255 (April 15), 114763. https://doi.org/10.1016/j.ecoenv.2023.114763.
Guo, M., Li, M., Cui, F., Ding, X., Gao, W., Fang, X., Chen, L., Wang, H., Niu, P., Ma, J., 2024a. Mtbe exposure may increase the risk of insulin resistance in male gas station workers. Environ. Sci. Process Imp. 26 (2), 334–343. https://doi.org/10.1039/d3em00491k.
Guo, M., Li, M., Cui, F., Wang, H., Ding, X., Gao, W., Fang, X., Chen, L., Niu, P., Ma, J., 2024b. Mediation effect of serum zinc on insulin secretion inhibited by methyl tert-butyl ether in gas station workers. Environ. Sci. Pollut. Res. Int. 31 (6), 8952–8962. https://doi.org/10.1007/s11356-023-31772-2.
Goyak, K.O., Sarang, S.S., Franzen, A., Borghoff, S.J., Ryman-Rasmussen, J.P., 2022. Adverse outcome pathway (AOP): α2u-globulin nephropathy and kidney tumors in male rats. Crit. Rev. Toxicol. 52 (5), 345–357. https://doi.org/10.1080/10408444.2022.2082269.
Guyton, K.Z., Rusyn, I., Chiu, W.A., Corpet, D.E., van den Berg, M., Ross, M.K., Christiani, D.C., Beland, F.A., Smith, M.T., 2018. Application of the key characteristics of carcinogens in cancer hazard identification. Carcinogen. 39 (4), 614–622. https://doi.org/10.1093/carcin/bgy031.
Greenough, R.J., McDonald, P., Robinson, P., Cowie, J.R., Maule, W., Macnaughton, F., Rushton, A., 1980. Methyl Tertiary Butyl Ether (Driveron) Three Month Inhalation Toxicity in Rats. Inveresk Research International, Edinburgh.
Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? Br. J. Pharmacol. 142 (2), 231–255. https://doi.org/10.1038/sj.bjp.0705776.
Hard, G.C., Banton, M.I., Bretzlaff, R.S., Dekant, W., Fowles, J.R., Mallett, A.K., McGregor, D.B., Roberts, K.M., Sielken Jr., R.L., Valdez-Flores, C., Cohen, S.M., 2013. Consideration of rat chronic progressive nephropathy in regulatory evaluations for carcinogenicity. Toxicol Sci. 132 (2), 268–275. https://doi.org/10.1093/toxsci/kfs305. Erratum. In: Toxicol Sci. 2013 Jun; 133(2):344.
Hard, G.C., Rodgers, I.S., Baetcke, K.P., Richards, W.L., McGaughy, R.E., Valcovic, L.R., 1993. Hazard evaluation of chemicals that cause accumulation of alpha 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ. Health Perspect. 99 (March), 313–349. https://doi.org/10.1289/ehp.9399313.
Hard, G.C., Betz, L.J., Seely, J.C., 2012. Association of advanced chronic progressive nephropathy (CPN) with renal tubule tumors and precursor hyperplasia in control F344 rats from two-year carcinogenicity studies. Toxicol. Pathol. 40 (3), 473–481. https://doi.org/10.1177/0192623311431948.
Haseman, J.K., 1983. A reexamination of false-positive rates for carcinogenicity studies. Fundam. Appl. Toxicol. 3 (4), 334–339. https://doi.org/10.1016/s0272-0590(83)80148-1.
He, Z., Xian, H., Tang, M., et al., 2021. DNA polymerase β may be involved in protecting human bronchial epithelial cells from the toxic effects induced by methyl tert-butyl ether exposure. Hum Exp Toxicol. 40 (12), 2135–2144. https://doi.org/10.1177/09603271211022788.
Health Canada, 2006. Guidelines for Canadian Drinking Water Quality: Guideline Technical Document — Methyl *Tertiary*-Butyl Ether (MTBE). Water Quality and Health Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. https://www.canada.ca/content/dam/canada/health-canada/migration/healthy-canadians/publications/healthy-living-vie-saine/water-mtbe-eau/alt/water-mtbe-eau-eng.pdf.
Hong, J.Y., Yang, C.S., Lee, M., Wang, Y.Y., Huang, W.Q., Tan, Y., Patten, C.J., Bondoc, F. Y., 1997. Role of cytochromes P450 in the metabolism of methyl tert-butyl ether in human livers. Arch. Toxicol. 71 (4), 266–269. https://doi.org/10.1007/s002040050386.
IARC (International Agency for Research on Cancer), 1999. Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 73. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Lyon, France. https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Chemicals-That-Cause-Tumours-Of-The-Kidney-Or-Urinary-Bladder-In-Rodents-And-Some-Other-Substances-1999#:~:text=Monographs%20Programme,or%20urinary%20bladder%20in%20rodents.
IARC (International Agency for Research on Cancer) Preamble, 2019. IARC Monographs Preamble–Preamble to the IARC Monographs (Amended January 2019. International Agency for Reaearch on Cancer, Lyon, France https://monographs.iarc.who.int/iarc-monographs-preamble-preamble-to-the-iarc-monographs/.
IARC (International Agency for Research on Cancer), 2024. Bias assessment in case-control and cohort studies for hazard identification. IARC Scientific Publication No. 171. Statistical Methods in Cancer Research, Volume V. https://publications.iarc.who.int/634.
Iavicoli, I., Carelli, G., Ardito, R., Cittadini, A., Sgambato, A., 2002. Methyl-tertiary-butyl ether (MTBE) inhibits growth and induces cell transformation in rodent fibroblasts. Anticanc. Res. 22 (4), 2173–2177.
IPCS (International Programme on Chemical Safety), 1998. Methyl tertiary-butyl ether. Environmental Health Criteria 206, International Programme on Chemical Safety, World Health Organization, Geneva. https://inchem.org/documents/ehc/ehc/ehc206.htm.
Kado, N.Y., Kuzmicky, P.A., Loarca-Piña, G., Moiz Mumtaz, M., 1998. Genotoxicity testing of methyl tertiary-butyl ether (MTBE) in the Salmonella microsuspension assay and mouse bone marrow micronucleus test. Mutat. Res. 412, 131–138. https://doi.org/10.1016/s1383-5718(97)00179-4.
Katoh, T., Arashidani, K., Kikuchi, M., Yoshikawa, M., Kodama, Y., 1993. Effects of methyl tertiary-butyl ether on hepatic lipid peroxidation in mice. Jap. J. Hyg. 48 (4), 873–878. https://doi.org/10.1265/jjh.48.873.
Khalili, L., Gholami, S., Ansari-Lari, M., 2015. Evaluation of offspring sex ratio, sex hormones and antioxidant enzymes following exposure to methyl tertiary butyl ether in adult male Sprague-Dawley rats. EXCLI J.14 (Jan 13), 75-82. Doi: 10.17179/excli2014-580.

Klimisch, H.-J., Andreae, M., Tillmann, U., 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regul. Toxicol. Pharmacol. 25 (1), 1–5. https://doi.org/10.1006/rtph.1996.1076.

Kozlosky, J., Bonventre, J., Cooper, K., 2013. Methyl tert butyl ether is anti-angiogenic in both in vitro and in vivo mammalian model systems. J. Appl. Toxicol. 33 (8), 820–827. https://doi.org/10.1002/jat.2737.

Lahr, J., Arts, G., Duquesne, S., Mazerolles, V., de Jong, F., Moermond, C., van der Steen, J., Alalouni, U., Baujard, E., van den Berg, S., Buddendorf, B., Faber, M., Mahieu, K., Montforts, M., Smit, E., van Spronsen, R., Swarowsky, K., Chaton, P.-F., Foldrin, J., Lambin, S., Pieper, S., 2023. Proposal of critical appraisal tools for the evaluation of ecotoxicology studies. EFSA Supp. Pub. 20 (3), 7787E. https://doi.org/10.2903/j.efsa.2023.EN-7787.

Leavens, T.L., Borghoff, S.J., 2009. Physiologically based pharmacokinetic model of methyl tertiary butyl ether and tertiary butyl alcohol dosimetry in male rats rased on binding to alpha2u-globulin. Toxicol. Sci. 109 (2), 321–335. https://doi.org/10.1093/toxsci/kfp049.

Li, D.M., Han, X.D., 2006. Evaluation of toxicity of methyl tert-butyl ether (MTBE) on mouse spermatogenic cells in vitro. Toxicol. Ind. Health. 22 (7), 291–299. https://doi.org/10.1177/0748233706070310.

Li, D., Liu, Q., Gong, Y., Huang, Y., Han, X., 2009. Cytotoxicity and oxidative stress study in cultured rat Sertoli cells with methyl tert-butyl ether (MTBE) exposure. Reprod. Toxicol. 27 (2), 170–176. https://doi.org/10.1016/j.reprotox.2008.12.004.

Li, D., Yin, D., Han, X., Han, X., 2007. Methyl tert-butyl ether (MTBE)-induced cytotoxicity and oxidative stress in isolated rat spermatogenic cells. Journal of Applied Toxicology : JAT 27, 10–17.

Li, D., Yuan, C., Gong, Y., Huang, Y., Han, X., 2008. The effects of methyl tert-butyl ether (MTBE) on the male rat reproductive system. Food Chem. Toxicol. 46 (7), 2402–2408. https://doi.org/10.1016/j.fct.2008.03.024.

Lin, K.K., Rahman, M.A., 1998. Overall false positive rates in tests for linear trend in tumor incidence in animal carcinogenic studies of new drugs. J. Biopharm. Statist. 8 (1), 1–15. https://doi.org/10.1080/10543409808835216.

Lington, A.W., Dodd, D.E., Ridlon, S.A., Douglas, J.F., Kneiss, J.J., Andrews, L.S., 1997. Evaluation of 13-week inhalation toxicity study on methyl t-butyl ether (MTBE) in Fischer 344 rats. J. Appl. Toxicol. 17 (Suppl 1), S37-44. Doi: 10.1002/(sici)1099-1263(199705)17:1+<s37::aid-jat409>3.3.co;2-h.

Ma, J., Chen, L., Song, D., Zhang, Y., Chen, T., Niu, P., 2017. SIRT1 attenuated oxidative stress induced by methyl tert-butyl ether in HT22 cells. Toxicol. Res. 6 (3), 290–296. https://doi.org/10.1039/c7tx00016b.

Mackerer, C.R., Angelosanto, F.A., Blackburn, G.R., Schreiner, C.A., 1996. Identification of formaldehyde as the metabolite responsible for the mutagenicity of methyl tertiary-butyl ether in the activated mouse lymphoma assay. Proc. Soc. Exper. Biol. Med. 212 (4), 338–341. https://doi.org/10.3181/00379727-212-44023.

Madia, F., Pilo, G., Worth, A., Corvi, R., Prieto, P., 2021. Integration of data across toxicity endpoints for improved safety assessment of chemicals: the example of carcinogenicity assessment. Arch. Toxicol. 95 (6), 1971–1993. https://doi.org/10.1007/s00204-021-03035-x.

Mansouri, K., Abdelaziz, A., Rybacka, A., Roncaglioni, A., Tropsha, A., Varnek, A., Zakharov, A., Worth, A., et al., 2016. CERAPP: Collaborative estrogen receptor activity prediction project. Environ. Health Perspect. 124 (7), 1023–1033. https://doi.org/10.1289/ehp.1510267.

Mansouri, K., Kleinstreuer, N., Abdelaziz, A.M., Alberga, D., Alves, V.M., Andersson, P.L., Andrade, C.H., Bai, F., et al., 2020. CoMPARA: collaborative modeling project for androgen receptor activity. Environ. Health Perspect. 128 (2), 27002. https://doi.org/10.1289/EHP5580.

Maronpot, R.R., Nyska, A., Foreman, J.E., Ramot, Y., 2016. The legacy of the F344 rat as a cancer bioassay model (a retrospective summary of three common F344 rat neoplasms). Crit. Rev. Toxicol. 46 (8), 641–675. https://doi.org/10.1080/10408444.2016.1174669.

Meek, M.E., Boobis, A., Cote, I., Dellarco, V., Fotakis, G., Munn, S., Seed, J., Vickers, C., 2014. New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. J. Appl. Toxicol. 34 (1), 1–18. https://doi.org/10.1002/jat.2949.

Meek, M.E.B., Wikoff, D., 2023. The need for good practice in the application of mechanistic constructs in hazard risk assessment. Toxicol. Sci. 194 (1), 13–22. https://doi.org/10.1093/toxsci/kfad039.

McGregor, D.B., Cruzan, G., Callander, R.D., May, K., Banton, M., 2005. The mutagenicity testing of tertiary-butyl alcohol, tertiary-butyl acetate and methyl tertiary-butyl ether in Salmonella typhimurium. Mutat. Res. 565 (2), 181–189. https://doi.org/10.1016/j.mrgentox.2004.10.002.

McGregor, D., 2006. Methyl tertiary-butyl ether: studies for potential human health hazards. Crit. Rev. Toxicol. 36 (4), 319–358. https://doi.org/10.1080/10408440600569938.

McGregor, D., 2010. Tertiary-butanol: a toxicological review. Crit Rev Toxicol. 40 (8), 697–727. https://doi.org/10.3109/10408444.2010.494249.

McKee, R.H., Vergnes, J.S., Galvin, J.B., Douglas, J.F., Kneiss, J.J., Andrews, L.S., 1997. Assessment of the in vivo mutagenic potential of methyl tertiary-butyl ether. J Appl. Toxicol. 17 (Suppl 1), S31–S36. https://doi.org/10.1002/(sici)1099-1263(199705)17:1+3.3.co;2-1.

Miller, M.J., Ferdinandi, E.S., Klan, M., Andrews, L.S., Douglas, J.F., Kneiss, J.J., 1997. Pharmacokinetics and disposition of methyl t-butyl ether in Fischer-344 rats. J. Appl. Toxicol. 17 (Suppl 1), S3–S. https://doi.org/10.1002/(sici)1099-1263(199705)17:1+<s3::aid-jat405>3.3.co;2-#.

Money, C.D., Tomenson, J.A., Penman, M.G., Boogaard, P.J., Lewis, R.J., 2013. A systematic approach for evaluating and scoring human data. Regulatory Toxicology and Pharmacology 66 (2), 241–247. https://doi.org/10.1016/j.yrtph.2013.03.011.

Moser, G.J., Wong, B.A., Wolf, D.C., Fransson-Steen, R.L., Goldworthy, T.L., 1996a. Methyl tertiary butyl ether lacks tumor-promoting activity in N-nitrosodiethylamine-initiated B6C3F1 female mouse liver. Carcinogen. 17 (12), 2753–2761. https://doi.org/10.1093/carcin/17.12.2753.

Moser, G.J., Wong, B.A., Wolf, D.C., Moss, W.R., Goldsworthy, T.L., 1996b. Comparative short-term effects of methyl tertiary butyl ether and unleaded gasoline vapor in female B6C3F1 mice. Fund. Appl. Toxicol. 31 (2), 173–183. https://doi.org/10.1006/faat.1996.0089.

Moser, G.J., Wolf, D.C., Sar, M., Gaido, K.W., Janszen, D., Goldsworthy, T.L., 1998. Methyl tertiary butyl ether-induced endocrine alterations in mice are not mediated through the estrogen receptor. Toxicol. Sci. 41 (1), 77–87. https://doi.org/10.1006/toxs.1997.2366.

Murphy, M.P., Bayir, H., Belousov, V., Chang, C.J., Davies, K.J.A., Davies, M.J., Dick, T. P., Finkel, T., et al., 2022. Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. Nat. Metab. 4 (6), 651–662. https://doi.org/10.1038/s42255-022-00591-z.

Nagatani, M., Kudo, K., Yamakawa, S., Ohira, T., Yamaguchi, Y., Ikezaki, S., Suzuki, I., Saito, T., Hoshiya, T., Tamura, K., Uchida, K., 2013. Occurrence of spontaneous tumors in the central nervous system (CNS) of F344 and SD rats. J. Toxicol. Pathol. 26 (3), 263–273. https://doi.org/10.1293/tox.26.263.

Najdegerami, I.H., Maghami, P., Sheikh-Hasani, V., Hosseinzadeh, G., Sheibani, N., Moosavi-Movahedi, A.A., 2017. Antichaperone activity and heme degradation effect of methyl tert-butyl ether (MTBE) on normal and diabetic hemoglobins. J. Mol. Recog. 30 (5), e2596.

Nihlén, A., Löf, A., Johanson, G., 1998. Experimental exposure to methyl tertiary-butyl ether. Toxicol. Appl. Pharmacol. 148 (2), 274–280. https://doi.org/10.1006/taap.1997.8333.

NTP (National Toxicology Program), 2018. Report on Carcinogens; Monograph on Antimony Trioxide. ISSN: 2331-267X. https://ntp.niehs.nih.gov/ntp/roc/monographs/antimony_final20181019_508.pdf.

Poet, T.S., Borghoff, S.J., 1997. In vitro uptake of methyl tert-butyl ether in male rat kidney: use of a two-compartment model to describe protein interactions. Toxicol. Appl. Pharmacol. 145 (2), 340–348. https://doi.org/10.1006/taap.1997.8193.

Prah, J., Ashley, D., Blount, B., Case, M., Leavens, T., Pleil, J., Cardinali, F., 2004. Dermal, oral, and inhalation pharmacokinetics of methyl tertiary butyl ether (MTBE) in human volunteers. Toxicol. Sci. 77 (2), 195–205. https://doi.org/10.1093/toxsci/kfh009.

Prescott-Mathews, J.S., Wolf, D.C., Wong, B.A., Borghoff, S.J., 1997. Methyl tert-butyl ether causes α2u-globulin nephropathy and enhanced renal cell proliferation in male Fischer-344 rats. Toxicol. Appl. Pharamcol. 143 (2), 301–314. https://doi.org/10.1006/taap.1996.8085.

Prescott-Mathews, J.S., Poet, T.S., Borghoff, S.J., 1999. Evaluation of the in vivo unteraction of methyl tert-butyl ether with a2u-globulin in male F-344 rats. Toxicol. Appl. Pharmacol. 157 (1), 60–67. https://doi.org/10.1006/taap.1999.8661.

Rao, G.N., 1997. New nonpurified diet (NTP-2000) for rodents in the National Toxicology Program's toxicology and carcinogenesis studies. J. Nutr. 127 (5 Suppl), 842S–846S. https://doi.org/10.1093/jn/127.5.842S.

Rice, J.M., Wilbourn, J.D. 2000. Tumors of the nervous system in carcinogenic hazard identification. Toxicol Pathol. 2000 28(1):202-14. doi: 10.1177/019262330002800125.

Reisfeld, B., de Conti, A., El Ghissassi, F., Benbrahim-Tallaa, L., Gwinn, W., Grosse, Y., Schubauer-Berigan, M., 2022. kc-hits: a tool to aid in the evaluation and classification of chemical carcinogens. Bioinformatics. 38 (10), 2961–2962. https://doi.org/10.1093/bioinformatics/btac189.

Ren, Q., Xie, X., Tang, Y., Hu, Q., Du, Y., 2021. Methyl tertiary-butyl ether inhibits THP-1 macrophage cholesterol efflux in vitro and accelerates atherosclerosis in ApoE-deficient mice *in vivo*. J. Environ Sci. 101 (March), 236–247. https://doi.org/10.1016/j.jes.2020.08.011.

Robinson, M., Bruner, R.H., Olson, G.R., 1990. Fourteen- and ninety-day oral toxicity studies of methyl teritary-butyl ether in Sprague-Dawley rats. J. Amer. Coll. Toxicol. 9, 525–540. https://doi.org/10.3109/109158190090787.

Rodriguez, V.I., Mammadova, J., Permuth, J.B., Luthra, A., Pena, L., Friedman, M., Dam, A., Cappelle, S., Malafa, M.P., Hallmon, C., Miranda, C., Mok, S.R.S., 2024. Elevated urinary levels of fungal and environmental toxins in patients with pancreatic ductal adenocarcinoma. J. Gastrointest. Cancer 6 (1), 4. https://doi.org/10.1007/s12029-024-01125-4.

Rota F, Conti A, Campo L, et al. Epigenetic and Transcriptional Modifications in Repetitive Elements in Petrol Station Workers Exposed to Benzene and MTBE. Int. J. Environ. Res. Public Health. 2018;15(4):735. Published 2018 Apr 12. doi:10.3390/ijerph15040735.

Saeedi, A., Omidi, M., Khoshnoud, M.J., Mohammadi-Bardbori, A., 2017a. Exposure to methyl tert-butyl ether (MTBE) is associated with mitochondrial dysfunction in rat. Xenobiot. 47 (5), 423–430. https://doi.org/10.3109/00498254.2015.1125040.

Saeedi, A., Fardid, R., Khoshnoud, M.J., Kazemi, E., Omidi, M., Mohammadi-Bardbori, A., 2017b. Disturbance of zinc and glucose homeostasis by methyl tert-butyl ether (MTBE); evidence for type 2 diabetes. Xenobiot. 47 (6), 547–552. https://doi.org/10.1080/00498254.2016.1201872.

Salimi, A., Vaghar-Moussavi, M., Seydi, E., Pourahmad, J., 2016. Toxicity of methyl tertiary-butyl ether on human blood lymphocytes. Environ. Sci. Pollut. Res. Int. 23 (9), 8556-8554. Doi: 10.1007/s11356-016-6090-x.

Sarhan, O.M., Jain, A., Mutwally, H.M.A., Osman, G.H., Yun Jung, S., Issa, T., Elmogy, M., 2019. Impact effect of methyl tertiary-butyl ether "Twelve months vapor inhalation study in rats. Biolog. 9 (1), 2. https://doi.org/10.3390/biology9010002.

Schoeb, T.R., McConnell, E.E., Juliana, M.M., Davis, J.K., Davidson, M.K., Linsey, J.R., 2009. Mycoplasma pulmonis and lymphoma in bioassays in rats. Vet. Pathol. 46 (5), 952–959. https://doi.org/10.1354/vp.08-VP-0240-S-COM.

Schneider, K., Schwarz, M., Burkholder, I., Kopp-Schneider, A., Edler, L., Kinsner-Ovaskainen, A., Hartung, T., Hoffmann, S., 2009. "ToxRTool", a new tool to assess the reliability of toxicological data. Toxicol. Lett. 189 (2), 138–144. https://doi.org/10.1016/j.toxlet.2009.05.013.

Sgambato, A., Iavicoli, I., De Paola, B., Bianchino, G., Boninsegna, A., Boninsegna, A., Bergamaschi, A., Pietroiusti, A., Cittadini, A., 2009. Differential toxic effects of methyl tertiary butyl ether and tert-butanol on rat fibroblasts in vitro. Toxicol. Ind. Health 25 (2), 141–151. https://doi.org/10.1177/0748233709104867.

Shen, S., Li, N., Ye, X., Huang, D., Qian, Y., Zheng, H., Dong, Z., Wan, X., 2023. Effect of tert-butyl acetate and ethyl butyrate on the dissolution of galbladder cholesterol stones. Chin. J. Hepatobil. Surg. 29 (3), 209–213.

Sills, R.C., Hailey, J.R., Neal, J., Boorman, G.A., Haseman, J.K., Melnick, R.L., 1999. Examination of low-incidence brain tumor responses in F344 rats following chemical exposures in National Toxicology Program carcinogenicity studies. Toxicol Pathol. 27 (5), 589–599. https://doi.org/10.1177/019262339902700513.

Silva, L.K., Espenship, M.F., Pine, B.N., Ashley, D.L., De Jesus, V.R., Blount, B.C., 2019. Methyl tertiary-butyl ether exposure from gasoline in the U.S. population, NHANES 2001-2012. Environ. Health Perspect. 127 (12), 127003. Doi: 10.1289/EHP5572.

Smith, M.T., Guyton, K.Z., Gibbons, C.F., Fritz, J.M., Portier, C.J., Rusyn, I., DeMarini, D. M., Caldwell, J.C., et al., 2016. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. Environ. Health Perspect. 124 (6), 713–721. https://doi.org/10.1289/ehp.1509912.

Smith, M.T., Guyton, K.Z., Kleinstreuer, N., Borrel, A., Cardenas, A., Chiu, W.A., Felsher, D.W., Gibbons, C.F., et al., 2020. The key characteristics of carcinogens: relationship to the hallmarks of cancer, relevant biomarkers, and assays to measure them. Cancer Epidemiol. Biomark. Prev. 29 (10), 1887–1903. https://doi.org/10.1158/1055-9965.EPI-19-1346.

Song, C., Zhang, Z., Chen, X., Zhang, Y., Wang, C., Liu, K., 2002. Study on three kinds of gasoline oxygenates-induced DNA damage in mice fibroblasts. Chin. J. Ind. Hyg. Occup. Dis. 20 (5), 362–364.

Steinbach, T.J., Maronpot, R.R. Hardisty, J.F., 2015. Human relevance of rodent Leydig cell tumors. In: Hamilton & Hardy's Industrial Toxicology. R.D. Harbison, Bourgeois, M.M. and Johnson, G.T., eds. Doi: 10.1002/9781118834015.ch109.

Swenberg, J.A., Lehman-McKeeman, L.D., 1999. alpha 2-Urinary globulin-associated nephropathy as a mechanism of renal tubule cell carcinogenesis in male rats. IARC Sci. Publ. 147, 95–118.

Tang, Y., Ren, Q., Wen, Q., Yu, C., Xie, X., Hu, Q., Du, Y., 2019. Effect of methyl tert-butyl ether on adipogenesis and glucose metabolism in vitro and in vivo. J. Environ. Sci. 85 (Nov), 208–219. https://doi.org/10.1016/j.jes.2019.06.015.

Thompson, C.M., Dewhurst, N., Moundous, D., Borghoff, S.J., Haws, L.C., Vasquez, M.Z., 2024. Assessment of the genotoxicity of tert-butyl alcohol in an in vivo thyroid comet assay. Environ. Mol. Mutagen. 65 (3–4), 129–136. https://doi.org/10.1002/em.22601.

Vergnes, J.S., Kintigh, W.J., 1993. Methyl Tertiary Butyl Ether Bone Marrow Micronucleus Test in Mice with Cover Letter Dated 111593. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/OTS0556627.xhtml.

Vergnes, J.S., Morabit, E.R., 1989. Methyl Tertiary Butly Ether Repeated Exposure Vapor Inhalation Study in Rats: In vivo Cytogenetic Evaluation (Final Report) with Cover Letter Dated 051889. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/OTS0528040.xhtml.

Wang, Y., Chen, C., Wu, T., Xu, J., Han, X., 2008. Methyl tert-butyl ether (MTBE) induced Ca(2+)-dependent cytotoxicity in isolated rabbit tracheal epithelial cells. Toxicol. In Vitro 22 (7), 1734-1741. Doi: j.tiv.2008.07.014.

Wang, H., Guo, M., Cui, F., et al., 2025. miR-18a-5p/PXR/SREBP2 was involved in MAFLD associated with methyl tert-butyl ether among petrol station workers. Liver Int. 45 (2), e16246. https://doi.org/10.1111/liv.16246.

Wikoff, D.S., Rager, J.E., Chappell, G.A., Fitch, S., Haws, L., Borghoff, S.J., 2019. A framework for systematic evaluation and quantitative integration of mechanistic data in assessments of potential human carcinogens. Toxicol. Sci. 167 (2), 322–335. https://doi.org/10.1093/toxsci/kfy279.

White Jr., K.L., Peachee, V.L., Armstrong, S.R., Twerdok, L.E., Clark, C.R., Schreiner, C. A., 2014. Health assessment of gasoline and fuel oxygenate vapors: immunotoxicity evaluation. Regul. Toxicol. Pharmacol. 70 (2 Suppl), S43–S47. https://doi.org/10.1016/j.yrtph.2014.04.010.

Williams, A.J., Grulke, C.M., Edwards, J., McEachran, A.D., Mansouri, K., Baker, N.C., Patlewicz, G., Shah, I., 2017. The CompTox chemistry dashboard: a community data resource for environmental chemistry. J. Cheminform 9 (1), 61. https://doi.org/10.1186/s13321-017-0247-6.

Williams, T.M., Cattley, R.C., Borghoff, S.J., 2000. Alterations in endocrine responses in male Sprague-Dawley rats following oral administration of methyl tert-butyl ether. Toxicol. Sci. 54 (1), 168–176. https://doi.org/10.1093/toxsci/54.1.168.

Williams, T.M., Borghoff, S.J., 2000. Induction of testosterone biotransformation enzymes following oral administration of methyl tert-butyl ether to male Sprague-Dawley rats. Toxicol. Sci. 57 (1), 147–155. https://doi.org/10.1093/toxsci/57.1.147.

Williams, T.M., Borghoff, S.J., 2001. Characterization of tert-butyl alcohol binding to a2u-globulin in F-344 rats. Toxicol. Sci. 62 (2), 228–235. https://doi.org/10.1093/toxsci/62.2.228.

Williams-Hill, D., Spears, C.P., Prakash, S., Olah, G.A., Shamma, T., Moin, T., Kim, L.Y., Hill, C.K., 1999. Mutagenicity studies of methyl-tert-butylether using the Ames tester strain TA102. Mut. Res. 446 (1), 15–21. https://doi.org/10.1016/s1383-5718(99)00137-0.

Xie, G., Hong, W.X., Zhou, L., Yang, X., Huang, H., Wu, D., Huang, X., Zhu, W., Liu, J., 2017. An investigation of methyl tert-butyl ether-induced cytotoxicity and protein profile in Chinese hamster ovary cells. Mol. Med. Rep. 16 (6), 8595–8604. https://doi.org/10.3892/mmr.2017.7761.

Yang, H., Kong, L., Zhao, J.S., 2005. DNA damage induced by methyl tertiary-butyl ether in vivo and in vitro. Chinese J. Ind. Hyg. Occup. Dis. 23 (5), 362–365.

Yuan, Y., Wang, H.F., Sun, H.F., Du, H.F., Xu, L.H., Liu, Y.F., Ding, X.F., Fu, D.P., Liu, K. X., 2007. Adduction of DNA with MTBE and TBA in mice studied by accelerator mass spectrometry. Environ. Toxicol. 22 (6), 630–635. https://doi.org/10.1002/tox.20295.

Zhu, Q., Zhu, S., Li, Q., Wang, Y., Zheng, D., Chen, C., Ruan, N., Chen, H., et al., 2022. Methyl tert-butyl ether inhibits pubertal development of Leydig cells in male rats by inducing mitophagy and apoptosis. Ecotoxicol. Environ. Saf. 232 (Mar), 113282. https://doi.org/10.1016/j.ecoenv.2022.113282.

Zhou, W., Ye, S., 1998. Study on the genotoxicity of methyl tertiary-butyl ether. J. Hyg. Res. 27 (5), 309–311.

Zhou, W., Huang, G., Zhang, H., 1999a. Effect of methyl tertiary butyl ether on the expression of proto-oncogenes and function genes. J. Hyg. Res. 28 (3), 137–138.

Zhou, W., Ye, S., Zhu, Y., 1999b. Effects of methyl tertiary butyl ether on liver tissue gene expression in rats. Chinese J. Prev. Med. 33 (2), 85–87.

Zhou, W., Yuan, D., Huang, G., Zhang, H., Ye, S., 2000a. Mutagenicity of methyl tertiary butyl ether. J Environ. Pathol. Toxicol. Oncol. 19 (1–2), 35–39.

Zhou, W., Huang, G., Zhang, H., Ye, S., 2000b. Effects of methyl tertiary butyl ether on cell cycle and cell apoptosis. Chinese J. Prev. Med. 34 (4), 221–223.