Reassessment of MTBE cancer potency considering modes of action for MTBE and its metabolites

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Abstract
A 1999 California state agency cancer potency (CP) evaluation of methyl tert-butyl ether (MTBE) assumed linear risk extrapolations from tumor data were plausible because of limited evidence that MTBE or its metabolites could damage DNA, and based such extrapolations on data from rat gavage and rat and mouse inhalation studies indicating elevated tumor rates in male rat kidney, male rat Leydig interstitial cells, and female rat leukemia/lymphomas. More recent data bearing on MTBE cancer potency include a rodent cancer bioassay of MTBE in drinking water; several new studies of MTBE genotoxicity; several similar evaluations of MTBE metabolites, formaldehyde, and tert-butyl alcohol or TBA; and updated evaluations of carcinogenic model(s) of action (MOAs) of MTBE and MTBE metabolites. The lymphoma/leukemia data used in the California assessment were recently declared unreliable by the U.S. Environmental Protection Agency (EPA). Updated characterizations of MTBE CP, and its uncertainty, are currently needed to address a variety of decision goals concerning historical and current MTBE contamination. To this end, an extensive review of data sets bearing on MTBE and metabolite genotoxicity, cytotoxicity, and tumorigenicity was applied to reassess MTBE CP and related uncertainty in view of MOA considerations. Adopting the traditional approach that cytotoxicity-driven cancer MOAs are inoperative at very low, non-cytotoxic dose levels, it was determined that MTBE most likely does not increase cancer risk unless chronic exposures induce target-tissue toxicity, including in sensitive individuals. However, the corresponding expected (or plausible upper bound) CP for MTBE conditional on a hypothetical linear (e.g., genotoxic) MOA was estimated to be $\sim 2 \times 10^{-5}$ (or 0.003) per mg MTBE per kg body weight per day for adults exposed chronically over a lifetime. Based on this conservative estimate of CP, if MTBE is carcinogenic to humans, it is among the weakest 10% of chemical carcinogens evaluated by EPA.

Keywords
cancer potency, cytotoxicity, DNA damage, epidemiology, formaldehyde, methyl tert-butyl ether, metabolism, mode of action, PBPK models, pharmacokinetics, risk assessment, tert-butyl alcohol, tumors

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1. Introduction

The human carcinogenic potency of methyl tert-butyl ether (MTBE) remains an open question more than a decade after California phased out its use based on a 1999 cancer risk assessment (CalEPA OEHHA, 1999). MTBE is a gasoline oxygenate additive that was used widely prior to phase-outs in the U.S., with state bans starting in 1999, and accelerating after Congress passed the Energy Policy Act of 2005 removing the oxygenate requirement for reformulated gasoline; a total of 25 states banned MTBE by 2007, with effective dates ranging from 2000 to 2009 (U.S. EPA 2007). MTBE is no longer used in significant quantities as a fuel oxygenate in the U.S., but ~1 million barrels/year continue to be manufactured for export to countries that still allow it as a gasoline additive (U.S. DOE 2014). A Working Group of the International Agency for Research on Cancer (IARC) that met in October 1998 to address MTBE carcinogenicity had concluded that human epidemiological evidence was inadequate, and that despite limited evidence for MTBE-induced tumors in experimental animals, MTBE was “not classifiable as to its carcinogenicity for humans (Group 3)” (IARC 1999). That conclusion was cited in the MTBE toxicity review by the World Health Organization (WHO) International Programme on Chemical Safety (IPCS), which concluded that “MTBE should be considered a rodent carcinogen” that “is not genotoxic and the carcinogenic response is only evident at high levels of exposure that also induce other adverse effects,” and that “available data are inconclusive and prohibit their use for human carcinogenic risk assessment until outstanding complications in their interpretation have been addressed” (WHO IPCS 1998). At about this time, the review group (RG) for the National Institute of Environmental Health Sciences (NIEHS) Report on Carcinogens was considering whether or not MTBE should be listed in the 9th Report. The first review group (RG1) supported listing (4 in favor and 3 against), while the second review group (RG2) did not support listing (3 in favor and 4 against). Ultimately, MTBE was not listed in the 9th or subsequent reports, further suggesting that available rodent cancer data were not strong enough for such a listing because observed kidney tumors in rats and liver tumors in mice may have arisen by mechanisms not relevant to humans, and that there were no supporting human data (National Toxicology Program [NTP] 1998). A European Union (EU) review committee later similarly concluded that there is “insufficient evidence for MTBE to be classified as a carcinogen” (EU 2002). The WHO did not establish a health-based value for MTBE in drinking water, citing earlier conclusions mentioned above (WHO IPCS 1998, IARC 1999), and its further conclusion that any health-based guideline for MTBE would be significantly higher than the 15-μg/L concentration at which MTBE is detected by odor among test- and order-sensitive human subjects (WHO 2005).

The U.S. Environmental Protection Agency (EPA) subsequently determined that data on lymphomas and leukemias obtained at the Ramazzini Institute that were used in the Office of Environmental Health Hazard Assessment (OEHHA) risk assessment are not reliable (U.S. EPA 2012a). New data have been published concerning MTBE and metabolite tumorigenicity, genotoxicity, and carcinogenic mode of action (MOA). There is consequently a need to reconsider the issue of cancer risks posed by low-level, environmental exposures to MTBE. MTBE continues to be detected in groundwater used for large and small water distribution systems at median detected concentrations on the order of ~10 μg/L affecting >1.5 million people, as well as in soil, at sites throughout the U.S. affected by gasoline leaks from underground storage tanks and other gasoline storage and distribution facilities, and continuing contamination is expected to persist for decades (U.S. EPA 2008). This paper addresses this issue by evaluating relevant available data and by providing refined estimates of MTBE carcinogenic potency characterized to be useful for a variety of decision-making contexts.

For MTBE exposure to increase human cancer risk, it must be a human carcinogen. Whether MTBE has human carcinogenic potential is a matter of hazard identification (NRC 1983), and this question is currently a matter of debate among toxicologists. Professional judgment in this regard can depend on the degree of conservatism (or tendency toward worst-case interpretations) that is incorporated into criteria applied to make this judgment, which in turn typically depends on the context of decisions to be made concerning related exposure and risk assessment (Bogen et al. 2009). Toxicological judgments made for the purpose of regulatory toxicology tend to be protective (err on the side of safety, within practical limits), while those made for the purpose of balancing costs, risks, and benefits must be predictive if the outcome of balancing is to be fair and impartial (NRC 1994, Bogen 2005, Bogen et al. 2009).

If MTBE (or any agent) is a human carcinogen, and if this agent increases the risk of cancer in linear proportion to dose at very low doses, then, by definition, increased cancer risk must nearly equal the product of dose and its carcinogenic potency (or increased risk per unit dose), and the aggregate risk posed by many such increased risks is expected to be well approximated by the sum of all the individual increased risks (NRC 1994, Supplementary Appendix I-1 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Under this assumption, it follows that a reasonable expectation of the magnitude of a potential risk of cancer, if any, posed by chronic human exposure—including whether such potential risk exceeds a de minimis level of risk—can be determined only using estimates of that exposure, of the dose resulting from that exposure, and of the carcinogenic potency of the agent to which people are exposed (NRC 1983, U.S. EPA 1986).
It is observed and expected that some chemical carcinogens, including some genotoxic chemical carcinogens (such as the MTBE metabolite, formaldehyde), may increase cancer risk with a dose–response that is highly nonlinear. It follows that there may be a cancer potency that is virtually zero at MTBE doses below those that induce cytotoxic effects observed in animals with MTBE-induced tumors. Let the probability $P_0$ denote the likelihood that this zero-potency assumption is true, and the probability $P_{\text{cancer}}$ denote the likelihood that MTBE is a human carcinogen. The value $1 – P_0$ is therefore the likelihood that the cancer potency of MTBE is greater than zero, which also is the chance that increased cancer risk for MTBE has a linear no-threshold (LNT) relationship to dose at very low doses. It follows that the following two approximations can be used to estimate the value of increased MTBE-related cancer risk fairly accurately, at all levels of risk less than $\sim 10\%$:

\begin{align}
\text{Increased Cancer Risk} &\approx P_{\text{cancer}} [(1 – P_0)Q] D, \\
\text{Increased Cancer Risk} &\approx Q_{\text{MOA}} D,
\end{align}

where $D$ denotes MTBE dose, and $Q$ denotes estimated MTBE cancer potency assuming an LNT MOA for any MTBE-induced increase in cancer risk, and the quantity $Q_{\text{MOA}} = (1 – P_0)Q$ denotes MTBE cancer potency estimated in a way that accounts for the likelihood of an LNT MOA for MTBE-induced tumors under the assumption that MTBE is a potential human carcinogen (that is, assuming that $P_{\text{cancer}} = 1$).

The specific purpose of this evaluation is to estimate the quantities $P_{\text{cancer}}$, $Q$, and $Q_{\text{MOA}}$, and so provide key inputs to an assessment of MTBE-related cancer risk. Doing this requires a consideration of MTBE and metabolite pharmacokinetics (absorption, distribution, metabolism, and excretion [ADME]), MTBE and metabolite genotoxicity (that is, the ability of the chemical to damage DNA, and so provide a basis for an LNT expectation that $Q$ may be greater than zero regardless of dose $D$), and MTBE and metabolite tumorigenicity and tumorigenic MOA. Based on these considerations, the human carcinogenic potency of MTBE was estimated based on available bioassay data and its likely MOAs both under protective and predictive conditions. The potential use of these new potency estimates in calculating cancer risks from MTBE exposures is demonstrated.

To support this study, literature searches were conducted for English-language publications concerning MTBE and its metabolites using names, synonyms, Chemical Abstracts Service (CAS) registry numbers, and several terms designed to optimize relevance of the captured publications. Searches were conducted initially in July 2012, and updated in April 2015. PubMed was searched for MEDLINE-indexed citations with limits applied as described below. Toxline was also utilized.

**MTBE.** Our PubMed search used the terms: (“methyl tert-butyl ether” OR MTBE OR TMBE OR “methyl tertiary-butyl ether” OR “methyl t-butyl ether” OR “t-tert-butyl methyl ether” OR “tert-butyl methyl ether” OR “tertiary-butyl methyl ether” OR “t-butyl methyl ether” OR 1634-04-4) AND (tox* OR safe* OR metab* OR absorb* OR eliminat* OR distribut* OR risk OR geno* OR carcino* OR tumo* OR kidney OR liver OR hepat* OR nephr* OR micro* OR chromo* OR mutat* OR adduct OR lesion OR lympho* OR leukemia* OR strand OR crosslink* OR cytogen*). For this and the following searches (unless otherwise indicated), the timeframe was limited to begin January 1, 2001, and English and “Other animal” filters were applied. 733 citations were returned.

**t-Butanol.** Our PubMed search used the terms: (“t-butyl alcohol” OR “tert-butyl alcohol” OR “t-butyl alcohol” OR “t-tert-butanol” OR “tertiary-butanol” OR “t-butanol” OR TBA OR 75-65-0 OR “1,1-dimethyl ethanol” OR “2-methyl-2-propanol”) AND (tox* OR safe* OR metab* OR absorb* OR eliminat* OR distribut* OR risk OR geno* OR carcin* OR tumo* OR kidney OR liver OR hepat* OR nephr* OR micro* OR chromo* OR mutat* OR adduct OR lesion OR lympho* OR leukemia* OR strand OR crosslink* OR cytogen*). Human epidemiology was not targeted in this search. 381 citations were returned.

**Formaldehyde.** Our PubMed search used the terms: formaldehyde AND (squamous OR cancer OR carcino* OR muta* OR genotox* OR chronic or crosslink OR adduct OR aneuploid* OR micronucle* OR chromosomal). The timeframe was limited to begin Jan 1, 2010, for non-human animals. 558 citations were returned.

**Formic acid.** Our Toxline search using the formic acid CAS registry number, which automatically captures synonyms, without date limits, yielded > 2,000 citations. Titles captured were searched for relevant key words, and relevant publications and review articles were all obtained and reviewed. Toxline was used in this case because PubMed returned too many citations to review (> 7,000), even including the limiting string of terms: tox* OR safe* OR metab* OR absorb* OR eliminat* OR distribut* OR risk OR geno* OR carcino* OR tumo* OR kidney OR liver OR hepat* OR nephr* OR micro* OR chromo* OR mutat* OR adduct OR lesion OR lympho* OR leukemia* OR strand OR crosslink* OR cytogen*.

**Methanol.** Our PubMed search focused on possible genotoxic or carcinogenic effects of methanol exposure. Search terms included methanol AND (geno* OR adduct OR muta* OR aberration OR exchange OR micronucle* OR lymphoma OR leukemia). Citations were limited to those in English and those published after the comprehensive review by Cruzan et al. (2009). 627 citations were returned. Using the slightly more broad search term string of “methanol and (adduct or geno* or muta*)”, 703 citations were returned. Adding more terms such as “chroma*” and “DNA” returned greater than 4,000 citations (too many to review feasibly).

Sections below refer to Supplementary Appendices A–D to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367, which present supporting data tables, methods of analysis used, and focused discussions of specific related issues addressed below. All these appendices appear in Supplemental/Online materials that accompany this paper, together with Supplementary Appendix E to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 that lists references cited in Supplementary Appendices A–D to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367.
2. MTBE, MTBE metabolites, and key related chemicals

Studies addressing the ADME, as well as available physiologically based pharmacokinetic (PBPK) models of MTBE and its metabolites, are discussed below. Glutathione metabolism has also been included due to its relevance in detoxification reactions. MTBE metabolites include tertiary butyl alcohol (tert-butyl alcohol, tert-butanol, or TBA), formaldehyde, formic acid, 2-methyl-1,2-propanediol, and alpha-hydroxyisobutyric acid. An analogous chemical, methanol, is included, because like MTBE it is metabolized to formaldehyde by a common, saturable, cytochrome P450 (CYP) mixed function oxidase enzyme pathway.

2.1 Absorption, distribution, metabolism, and excretion

2.1.1 MTBE

MTBE (CAS No. 1634-04-4) is absorbed via inhalation, ingestion, and dermal uptake. Both human and animal ADME data are available for MTBE. MTBE is metabolized to formaldehyde and TBA. Formaldehyde is further metabolized rapidly to formate (which is largely incorporated in the one-carbon pool) and to CO2, and TBA is further metabolized to α-hydroxyisobutyric acid, 2-methyl-1,2-propanediol, TBA conjugates, and to a minor extent, acetone (see below, and Section 2.1.3). Under most experimental conditions, the major part of MTBE in the body is excreted as urinary metabolites, and less than half is exhaled unchanged; however, if the uptake rate is high, the opposite is true.

MTBE is readily absorbed during inhalation exposure (Buckley et al. 1997, Miller et al. 1997, Nihlén et al. 1999), and ingestion (Miller et al. 1997, Prah et al. 2004). Prah et al. (2004) studied adult volunteers who either inhaled an MTBE concentration of 3.1 ppm in air for one hour, drank 2.78 mg of MTBE mixed in 250 mL of Gatorade®, or were dermally exposed to 51.3 µg/mL of MTBE in tap water warmed to 39°C for one hour. MTBE and TBA in exhaled breath were measured during and after each experimental exposure scenario, and in blood after each experimental exposure scenario. Data sets for MTBE concentration in exhaled breath and in blood were each fit to a two- or a three-compartment biokinetic model, and three-compartment models were found to provide the best fits. These fits were integrated to estimate total MTBE mass exhaled versus that “retained” in the body. (Here and throughout this paper, “retained” is used as a term of convenience intended to mean “not exhaled.”) Neither MTBE nor any of its metabolites are retained permanently in the body.) From these calculations, Prah et al. (2004) estimated that 47.2% of the total inhaled dose (26.6% of all MTBE that was “retained” in the body during inhalation), 53% of the oral MTBE dose, and 63% of the calculated dermally absorbed dose were excreted as MTBE in exhaled breath, and the remaining MTBE was retained in the body until it was metabolized. Based on observed route-specific ratios of TBA to MTBE concentration in blood over time, oral MTBE exposure to humans was concluded to produce significantly greater metabolism of MTBE to TBA than by other routes of exposure (Prah et al. 2004).

Metabolism of MTBE and other fuel-additive oxygenates was studied by Dekant et al. (2001) in humans and rats after inhalation of 4 and 40 ppm of MTBE for 4 h, and in humans who ingested 5 or 15 mg MTBE in water. In rats and humans, clearance from blood after inhalation exposure occurred with a half-time of <7 h, and all urinary metabolites (including primarily 2-hydroxyisobutyrate) were eliminated with half-times of <20 h. After the oral exposures in this study, hepatic first-pass metabolism of MTBE was not observed and a significant part of each administered oral dose was cleared by exhalation. In ddY mice that were administered 50, 100, or 500 mg/kg MTBE by intraperitoneal (i.p.) injection, dose-dependent percentages (23.2, 37.6, or 69.0%, respectively) of the administered MTBE were exhaled unchanged, suggesting that metabolic saturation occurs in this species at increasing MTBE doses (Yoshikawa et al. 1994). Using a PBPK model for MTBE in rats (further discussed in Section 2.2), Borghoff et al. (2010) estimated that adult male rats exposed for 91 days to 0.5–15 mg/mL of MTBE in drinking water, to 250–1,000 mg/kg of MTBE by gavage 5 days/week, and to 400–8,000 ppm of MTBE in air 5 days/week for 6 h/day, exhaled 67–83%, 81–88%, and 80–93% of the MTBE delivered via these exposure pathways, respectively.

In vivo rates of dermal uptake of MTBE into human skin that was immersed in a heated (39 or 32°C) dilute aqueous MTBE solution for 30 or 60 min have been measured using a biokinetic method, employing data on MTBE concentrations measured in exhaled breath (Prah et al. 2004), and by the disappearance method, by which net loss of MTBE is measured from an exposure solution that exceeds any loss observed from the same solution placed in a control exposure chamber in which no skin is immersed (Fan et al. 2007). Prah et al. (2004) estimated a dermal permeability coefficient of 0.028 cm/h for MTBE based on data from their study, and the corresponding value measured by Fan et al. (2007) was 0.109 cm/h, where each of these estimates represents an “effective” or time-weighted average (TWA) permeability coefficient measured under short-term, non-steady-state conditions. The permeability coefficient measured by Fan et al. (2007) is one of 18 such measures obtained for different dilute aqueous organic chemicals by the in vivo disappearance method that are all well predicted (to within 3-fold) by a single physicochemical regression model (Bogen 2013a).

Following absorption, MTBE is distributed throughout the body, has a blood:air partition coefficient of approximately 11.5, and is 7–10 times more soluble in fat than in blood (Nihlén et al. 1995, Borghoff et al. 1996, Imbriani et al. 1997). Solubilities in the rat liver and muscle are rather similar to those in blood (Borghoff et al. 1990, 1996). However, in the male rat kidney, MTBE concentration was observed to be six times greater than in blood due to alpha2u-globulin binding, which (as discussed in Section 5.2.1) is specific to the male rat and not relevant to humans (Borghoff et al. 1990, Prescott-Mathews et al. 1997, Prescott-Mathews et al. 1999).

The metabolism of MTBE is illustrated in Figure 1. In vivo studies of MTBE metabolism in rats (Miller et al. 1997, Bernauer et al. 1998, Amberg et al. 1999) and humans (Nihlén et al. 1999, Amberg et al. 1999, Nihlén and Johanson 1999) indicate qualitatively similar overall patterns of metabolism. The metabolic pathways involve oxidative demethylation of MTBE to formaldehyde and TBA by microsomal (cytochrome P450 or CYP) enzymes: in humans most actively
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by CYP2A6 with a minor contribution of CYP3A4 at low MTBE concentrations (Hong et al. 1997a, 1999a, 2001, Le Gal et al. 2001, 2003, Shamsipur et al. 2012), in rats (which do not have CYP2A6) by CYP2B1 and to a minor extent by CYP2E1 (Brady et al. 1990, Hong et al. 1997b; Turini et al. 1998), and in mice only negligibly by CYP2E1 (Hong et al. 1999b). Cytochrome P450 enzymes are relatively highly concentrated in rat nasal mucosal compared with liver tissue (Longo et al. 1986). These enzymes (including CYP2A6) are also present in primate and human nasal mucosa, though at concentrations or levels of expression relatively lower than those in liver (Longo et al. 1992, Thornton-Manning et al. 1997, Bogdanffy and Keller 1999, Koskela et al. 1999). In Sprague-Dawley rats, olfactory mucosa exhibited 46-fold greater MTBE-metabolizing activity than liver tissue (Hong et al. 1997b). Human liver microsomes were determined to exhibit a 24-fold variation of CYP2A6 catalytic activities for MTBE, and a 68-fold variation in CYP2A6 content (Le Gal et al. 2003).

In most studies with rats and humans, after inhalation exposure, more than half of the MTBE “retained” in the body (here and below meaning not exhaled, as previously noted) was biotransformed to urinary metabolites, and less than half was exhaled unchanged (Miller et al. 1997, Nihlén et al. 1999, Amberg et al. 1999, Dekant et al. 2001); however, in rats exposed to a high level via inhalation (8,000 ppm, 6 h), nearly all of the inhaled dose was exhaled, indicating metabolic saturation (Miller et al. 1997), consistent with the corresponding PBPK model predictions for the rat (Borghoff et al. 2010). In rats administered MTBE by intravenous (i.v.) injection, 60% of the radioactivity was exhaled, and 35% was found in urine (Miller et al. 1997). In dermal exposures, the fractions found in expired air and urine were equal (Miller et al. 1997).

The main urinary metabolites of MTBE detected in humans and rats were \( \alpha \)-hydroxyisobutyric acid accounting for about 70% of all urinary metabolites (Amberg et al. 1999), together with 2-methyl-1,2-propanediol and one or more TBA conjugates as other major metabolites, as well as some TBA (Amberg et al. 1999, Bernauer et al. 1998). After three male and three female human volunteers were exposed to 4 or 40 ppm MTBE for 4 h, there was wide inter-individual variation in \( \alpha \)-hydroxyisobutyric acid excretion, no significant difference by sex and marked pre-exposure background levels of \( \alpha \)-hydroxyisobutyric acid were detected (Amberg et al. 1999). Background levels of \( \alpha \)-hydroxyisobutyric acid are apparently due to endogenous formation (Liebich and Forst 1984). Using a method they developed to determine MTBE and TBA in human urine, Lee and Weisel (1998) found that of the MTBE inhaled by a human subject exposed to 1 ppm MTBE in air for 10 min, 0.9% was detected as unchanged urinary MTBE and 2.6% as TBA in urine within 10 h after exposure. Acetone was detected (at < 0.1% of administered dose) in exhaled breath of human volunteers who ingested 5 or 15 mg \([^{13}\text{C}]\)-MTBE in water (Amberg et al. 2001).

Comparison of the tentative metabolic clearance data between rats and humans suggests that the metabolic capac-

Figure 1. MTBE-related metabolism (adapted from Black 1985, ATSDR 1996, Bernauer et al. 1998, Amberg et al. 1999, EU 2002, NTP 2002, Stover 2009, U.S. EPA 2010). ADH = alcohol dehydrogenase, FDH = formaldehyde dehydrogenase (ADH3), GSH = reduced glutathione, CYP = cytochrome p450; THF = tetrahydrofolate.
ity is higher in rats, as also indicated by in vitro studies with hepatic microsomal preparations (EU 2002). Studies conducted so far with a limited number of human volunteers have not uncovered any remarkable inter-individual differences in metabolic clearance, and no indications of metabolic saturation, up to 75 ppm MTBE in inhaled air (Nihlén et al. 1999). MTBE had been speculated to be able to induce its own metabolism based on its similarity to other agents that induce hepatic microsomal P450 activity (Brady et al. 1990). Moser et al. (1996a) showed that in female B6C3F1 mice exposed by inhalation to 8,000 ppm of MTBE for 3 or 21 days CYP content was increased (significantly, by 2- to fold, after 21 days), microsomal enzyme activities were significantly increased (by 5- to 14-fold) as measured by 7-pentoxyresorufin-O-dealkylase or PROD activity after each exposure period, and likewise significantly increased (by 2- to 3-fold) as measured by 7-ethoxyresorufin-O-deethylase or EROD activity after each exposure period. The MTBE metabolite TBA is also able to induce its own metabolism (Section 2.1.3), and both types of autoinduced metabolism may involve common mechanisms.

MTBE and its metabolites have been shown to be eliminated rapidly in rodents (Yoshikawa et al. 1994, Miller et al. 1997), and have also been shown to exhibit a similarly rapid phase of clearance from humans at least initially (Nihlén et al. 1999, Amberg et al. 1999, Dekant et al. 2001, Prah et al. 2004). Pulmonary MTBE elimination measured after i.p. administration of 100 or 500 mg/kg of MTBE in ddY mice exhibited half-time ($t_{1/2}$) values of 45–80 min (Yoshikawa et al. 1994). Monophasic MTBE elimination with a $t_{1/2}$ of about 30 min was observed from F344 rat plasma after i.v., inhalation, or oral exposure (Miller et al. 1997, Amberg et al. 1999), whereas after dermal exposure to MTBE a larger initial $t_{1/2}$ value of 1.8–2.3 h that was observed is consistent with delayed systemic uptake via percutaneous absorption (Miller et al. 1997). After MTBE exposures to human volunteers by different routes, several studies examined the kinetics of MTBE elimination from exhaled air and/or from blood (Prah et al. 2004 tabulates these results by exposure route). For example, by inhalation, other estimated phase-specific $t_{1/2}$ values were 0.8 min, 12 min, 1.5 h, and 21 h (Nihlén et al. 1999); 1.3 and 2.4 h (Amberg et al. 2001, Dekant et al. 2001); and 1.9 min, 59 min, and 5.2 h (Prah et al. 2004). After human oral exposure to MTBE, reported phase-specific $t_{1/2}$ values were approximately 1.7–1.8 and 7.0–8.1 h (Amberg et al. 2001, Dekant et al. 2001, Prah et al. 2004). After human dermal exposure to MTBE, reported phase-specific $t_{1/2}$ values were approximately 2.1 and 6.7 h (Prah et al. 2004). Relatively rapid elimination of MTBE and its metabolites observed indicates that MTBE and its metabolites do not accumulate substantially either in rodents or humans after acute or intermittent exposures.

### 2.1.3 TBA

TBA (CAS No. 75-65-0) is a metabolite of MTBE (Figure 1). TBA absorption per se is not directly related to the present study, which concerns TBA as a metabolite of administered MTBE. Studies of MTBE and TBA metabolism in humans and rats have shown similar metabolic patterns, and identified 2-hydroxyisobutyrate in urine as the major common metabolite, with lesser amounts of methyl-1,2-propanediol, conjugated TBA, and unconjugated TBA appearing in urine (Bernauer et al. 1998, Amberg et al. 1999). Acetone was detected as a minor metabolite in urine from Long Evans and Sprague-Dawley rats administered a 1-g/kg dose of aqueous [13C]-radiolabeled TBA by i.p. injection (Baker et al. 1982), in urine collected from rats exposed by inhaling 2,000 ppm of [13C]-TBA for 6 h (Bernauer et al. 1998), and (at <0.1% of administered dose) in exhaled breath of human volunteers who ingested up to 5 or 15 mg of [13C]-MTBE in water (Amberg et al. 1999, see Section 2.1.1). In 11 rats each treated with an i.p. acetone dose of 0.75–2.0 g/kg, seven rats excreted 0.5–9.5% of the administered molar dose in urine following TBA, but four rats did not produce any detectable acetone (Bernauer et al. 1998). By $^{13}$C NMR spectrum analysis, the chemicals 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate were identified as metabolites of [13C]-radiolabeled TBA in urine collected from male and female F344 rats that inhaled 2,000 ppm of [13C]-radiolabeled TBA for 6 h and in the urine of one human volunteer orally administered 400 mg of [13C]-radiolabeled TBA (5 mg/kg).
(Bernauer et al. 1998), consistent with the identification of these chemicals as urinary metabolites from rats and humans exposed to $^{13}$C-radiolabeled MTBE (Section 2.1.1). In the urine of the human volunteer administered 5 mg/kg of $^{13}$C-radiolabeled TBA orally, TBA, TBA glucuronide, and one other unidentified metabolite were also detected (Bernauer et al. 1998). In humans, pre-test urine samples were found to contain a significant amount of 2-hydroxyisobutyrate and lower background levels of TBA and methyl-1,2-propanediol, indicating that natural sources of TBA and these two TBA metabolites exist (Amberg et al. 1999). Conjugation of TBA in humans is mainly through glucuronide conjugation with only a trace amount of sulfate conjugate in humans (Bernauer et al. 1998). In rats, the sulfate and glucuronide conjugates were found to be the major and minor conjugated metabolites, respectively (Bernauer et al. 1998).

The $t_{1/2}$ of 1.8 h for elimination of TBA from rat plasma after 15 daily exposures to 400 ppm of MTBE for 6 h/day is approximately half that (3.3 h) observed after a single such inhalation exposure (Miller et al. 1997). Values of $t_{1/2}$ ranged from 2.9 to 5 h for urinary excretion of primary urinary MTBE metabolites (TBA, 2-methyl-1,2-propanediol, and $\alpha$-hydroxyisobutyric acid) in F344 rats, and from 7.8 to 17 h in human volunteers (Amberg et al. 1999). Approximate first-order elimination of TBA from blood was estimated to have a $t_{1/2}$ of 9.1 h after rats were administered 1 g/kg of TBA by i.p. injection (Baker et al. 1982). A study of alcohol metabolism after alternating inhalation and i.p. dosing showed that TBA, like MTBE (Section 2.1.1), induces its own metabolism and that TBA is rapidly cleared (McComb and Goldstein 1979). TBA was not detected in blood 8–9 h after a single 600-mg/kg i.p. dose of TBA was administered to each of 9 male Swiss-Webster mice. After these mice subsequently inhaled 1.1–3.1% TBA in air for 3 days (attaining a mean blood TBA level of 590 mg/L), no TBA was detected in blood after an observation period of only 3 h. After the same mice were then administered a 600-mg/kg i.p. dose of TBA, again no TBA could be detected in blood 3 h later (McComb and Goldstein 1979).

In humans exposed to 4.5 or 40 ppm of MTBE via inhalation for 4 h, the $t_{1/2}$ value estimated for TBA in blood was 5–10 h (Nihlén et al. 1999, Amberg et al. 1999), and the percent of TBA recovered in the urine was 0.49–0.77% of the MTBE dose (Nihlén et al. 1999). In six human volunteers exposed to 5 and four weeks later to 15 mg of MTBE by ingestion (Amberg et al. 2001), the reported corresponding average $t_{1/2}$ values for TBA in blood do not differ significantly ($p = 0.78$, by t-test using data reported in Table 1 of that study), and the overall average $t_{1/2}$ value for TBA in blood, ± 1 standard deviation (SD), from this study was 8.3 ± 1.9 h.

### 2.1.4 Formaldehyde

The MTBE metabolite formaldehyde (CAS No. 55-00-0) is clearly produced endogenously by enzymatic and nonenzymatic pathways or as a detoxification product of xenobiotics during cellular metabolism, which (together with dietary sources) normally produces low formaldehyde concentrations in all tissues, cells, and bodily fluids (ATSDR 1999). Endogenous formaldehyde concentrations in rat, monkey, and human blood are approximately 1–2 $\mu$g/mL or about 0.1 mM (Heck et al. 1985, Casanova et al. 1988, NRC 2011), and background concentrations in the liver and nasal mucosa of the rat are 2–4 times those in the blood (Heck et al. 1982). Endogenous formaldehyde concentrations in tissues (~0.05–0.4 mM) are similar to formaldehyde concentrations that induce genotoxicity and cell killing under in vitro conditions (Heck et al. 1982, Heck and Casanova 2004), and are 5–100-fold less than normal cellular glutathione concentrations (Section 2.1.2).

Formaldehyde is absorbed by inhalation and by ingestion, whereas dermal uptake of formaldehyde is limited by the reaction with surface macromolecules and evaporation (U.S. EPA 2010). Upon inhalation, the majority of formaldehyde is absorbed in the upper respiratory tract, with approximately 90% of nasally inhaled formaldehyde estimated to be absorbed by nasal tissues at resting rates of human nasal inhalation (Kimbell et al. 2001). At physiological temperatures and pH (such as in tissues and blood), formaldehyde exists in equilibrium with its chemically favored and relatively less reactive hydrated form, methanediol (H$_2$C(OH)$_2$), and is subject to metabolism (discussed below) as well as to urinary and respiratory excretion (U.S. EPA 2010, NRC 2011).

Inhalation studies in several rats, rhesus monkeys, and humans indicate that exposure to formaldehyde does not result in elevated levels of formaldehyde in blood (Heck et al. 1982, 1983, 1985, Heck and Casanova-Schmitz 1984, Casanova et al. 1988, U.S. EPA 2010). Following exposure to radiolabeled formaldehyde, radioactivity was very high in the nasal mucosa but was also extensively distributed to various tissues, although it was not possible to determine whether detected radiolabel was free formaldehyde, reversibly bound formaldehyde, irreversibly bound formaldehyde, formate, or in the one-carbon pool of cellular metabolites that are subject to DNA incorporation (Stover 2009, U.S. EPA 2010) (Figure 1). The portion of the formaldehyde that enters the circulation does so bound to macromolecules or by incorporation of the radiolabel into the one-carbon pool (U.S. EPA 2010). DNA–protein crosslinks (DPX), as well as protein–protein crosslinks, are created when formaldehyde reacts covalently with amino or imino groups by nucleophilic attack of the carbonyl carbon of formaldehyde to form a Schiff base, which then undergoes reaction with another amino group on a second DNA base or protein to complete the crosslink (Barker et al. 2005). DPX provides a surrogate measure of the intracellular concentration of formaldehyde (Hernandez et al. 1994, U.S. EPA 2010). Using this approach, labeling in the nasal mucosa was determined to be due to both covalent binding and metabolic incorporation. In contrast, radiolabel detected in bone marrow was found to be due entirely to metabolic incorporation and not due to covalent binding via adduct formation (Casanova-Schmitz and Heck 1983, Casanova-Schmitz et al. 1984a, b).

Once generated as a metabolite of MTBE, formaldehyde undergoes further metabolism primarily by the glutathione-dependent formaldehyde dehydrogenase (FDH) enzyme, also referred to as alcohol dehydrogenase 3 (ADH3) (Liu et al. 2001, 2004, Høgg et al. 2003, Hedberg et al. 2003, Thompson et al. 2009) (Figure 1). The nonenzymatic product of formaldehyde with glutathione, $S$-(hydroxymethyl) glutathione (HMGSH), is the major form of formaldehyde seen in vivo (Sanghani et al. 2000, U.S. EPA 2010). The first metabolic pathway involves conversion of free formaldehyde to formate by the so-called
“low-K_m” (K_m = 400 μM) mitochondrial FDH (or ALD3). The second pathway involves a two-enzyme system that converts HMGSH to the intermediate S-formylglutathione, which is subsequently oxidatively metabolized to formate and GSH by S-formylglutathione hydrolase (U.S. EPA 2010). Formate is then incorporated into the one-carbon pool, metabolized to CO_2 and water, incorporated into a variety of larger molecules including purines used to synthesize cellular DNA, or excreted in the urine as a sodium salt (McMartin et al. 1979, Heck et al. 1983, Gottschling et al. 1984, Stover 2009) (Figure 1). Consistent with this metabolic scheme, after acute exposure to [^{14}C]-formaldehyde via inhalation or ingestion, the majority of administered radiolabel is detected in expired [^{14}C]-carbon dioxide (^{14}CO_2), whereas oral absorption of [^{14}C]-formaldehyde in rats results in 40% elimination as ^{14}CO_2, with 10% excretion in urine, 1% excretion in feces, and much of the remaining 49% retained in the carcass due presumably to metabolic incorporation (Heck et al. 1983, IARC 2012). However, no study has yet confirmed that HMGSH is generated \( \textit{de novo} \), either by MTBE-exposed mammalian cells \( \textit{in vitro} \), or (for example, systemically or in liver) in MTBE-exposed animals or humans.

### 2.1.5 Formic acid

Formic acid (CAS No. 64-18-6), the ionized form of which is referred to as formate is a chemical we presume to be generated during MTBE metabolism because it is a known metabolite of formaldehyde (Section 2.1.4). No study has yet confirmed that formate is generated \( \textit{de novo} \), either by MTBE-exposed mammalian cells \( \textit{in vitro} \), or (for example, systemically or in liver) in MTBE-exposed animals or humans. However, \( \textit{in vitro} \) results discussed in Section 3.1 were interpreted to suggest that the intracellular rate of formaldehyde production from MTBE was slow relative to the rate of formaldehyde oxidation to formate, which subsequently was incorporated into the one-carbon pool and thence into purines, DNA, and RNA (Figure 1). Formic acid is readily absorbed from the gastrointestinal tract, lungs, intact skin, and urinary bladder (NTP 1992). Hanzlik et al. (2005) examined the oral absorption of calcium formate in humans and report maximum absorption at 60 minutes (min) post-dose. Absorption then declined monoelexponentially, with an average half-life of 59 ± 7 min and returned to baseline values by 225 min post-dose, indicating that bioaccumulation is not likely. In several species including rats, monkeys, and humans, the t½ of sodium formate, the conjugate base salt of formic acid, ranged from 12 to 55 min, and its distribution was throughout body water (NTP 1992). As noted in Section 2.1.4, absorbed formic acid is oxidized to CO_2 and H_2O (Martin-Amat et al. 1978, Eells et al. 1981, 1983, NTP 1992) (Figure 1). Oxidation of formate occurs by folate-dependent and catalase-peroxidative mechanisms that occur primarily in the liver, although intestinal mucosa, lungs, kidney, erythrocytes, and spleen also contribute (Plaut et al. 1950, Stedman and Welsh 1989, Tephly 1991, Stover 2009).

Studies with perfused rat livers indicate that hepatic metabolism is sufficient to account for all of the formate oxidation observed \( \textit{in vivo} \) (Damian and Raabe 1996), although other tissues may also contribute (Plaut et al. 1950, Tephly 1991). Rodents and dogs metabolize formate much more efficiently than primates or humans (Hanzlik et al. 2005). The rate of formate oxidation to CO_2 in monkeys was markedly lower than that in rats (McMartin et al. 1979). Although the rate of oxidation was dose dependent in both species, metabolism in monkeys proceeded at a rate approximately one-half that measured in rats. The difference in the rate of formic acid oxidation seems to depend on hepatic concentrations of the essential coenzyme tetrahydrofolate (THF) that is synthesized from dietary folic acid (vitamin B_9) (Johlin et al. 1987, Nair and Andersen 1997). The rate of formate metabolism is determined primarily by THF availability (NTP 2002).

On i.p. or oral administration of ^{14}C-formate to animals, exhalation of ^{14}CO_2 commences almost immediately at rates that are dose proportional and with saturation, clearly observable at very high doses (Sperling et al. 1953, Hanzlik et al. 2005). Exhalation of ^{14}CO_2 accounts for up to 80% of administered doses of ^{14}C-formate, with most of this being recovered in the first few hours, even after oral administration (Sperling et al. 1953, Palese and Tephly 1975). Except at very high doses, the urinary excretion of formate is low, around 2–7% of the administered dose (Sperling et al. 1953, Malorny 1969, Hanzlik et al. 2005) and is limited by a renal transport system that recovers formate and a proton in exchange for chloride and sodium (Hanzlik et al. 2005). Because of endogenous metabolic incorporation of formate into tissue components, the carcass retains up to 10% of the dose of ^{14}C-formate even after eight days (Sperling et al. 1953).

Excretion of formic acid also is influenced by the amount administered; 8–9% was excreted unchanged by dogs given a 1-g oral dose, as compared with 65% excreted by dogs given a 5-g dose (Hanzlik et al. 2005). Formate from orally administered calcium formate is absorbed rapidly, distributed rapidly, and eliminated rapidly; therefore, accumulation of formate is not expected (Hanzlik et al. 2005).

### 2.1.6 Methanol

Methanol (CAS No. 67-56-1) is relevant to this analysis because, like MTBE, it is metabolized in the body to formaldehyde (Section 2.1.4). Methanol occurs naturally in the human body as a product of metabolism and through intake of fruits, vegetables, and alcoholic beverages (WHO IPCS 1997, NTP 2002). Background blood methanol levels in humans have been measured and reported to range from 0.6 to 2.6 mg/L with restricted diets, and otherwise are typically <3.5 mg/L (NTP 2002, U.S. EPA 2011). Methanol is absorbed rapidly following inhalation, ingestion, and dermal contact, and the absorption capabilities do not appear to differ substantially across mammalian species (WHO IPCS1997) or age (Steigink et al. 1983). Methanol distributes rapidly and uniformly to all organs and tissues in direct relation to their water content, with an overall volume of distribution of approximately 0.6 L/kg (HEI 1987, WHO IPCS1997). Tissue:blood concentration ratios for methanol are predicted to be similar via different exposure routes, although the kinetics will vary depending on exposure route and timing (NTP 2002).

In mammals, methanol is metabolized in a series of oxidation steps to sequentially form formaldehyde, formate, and carbon dioxide (Figure 1). Methanol can be oxidized to formaldehyde through three different pathways within the liver, although two
are of primary importance. In primates, alcohol dehydrogenase catalyzes the metabolism of methanol to formaldehyde, whereas in rodents, the catalase pathway performs this function. Despite this difference, this first metabolic step proceeds at similar rates in non-human primates and rats. FormFDH (ADH3) is found in liver, brain, and erythrocytes and catalyzes a reaction of formaldehyde with reduced glutathione to form S-formylglutathione, which subsequently hydrolyzes in the presence of glutathione thiolase to formic acid and reduced glutathione. Formate is oxidized primarily to carbon dioxide and water in mammals through a THF-dependent pathway (see Sections 2.1.4, 2.1.5; Figure 1).

After methanol is distributed in the body, it is either directly excreted in urine and exhaled breath or metabolized in the liver. Clearance from circulation in humans following low-level exposures follows first-order kinetics, with a half-time of ∼2.5–3 h (NTP 2002). At higher doses, the elimination becomes saturated. The kidney appears to exert no active control over urinary methanol concentration. Exhalation levels and excretion by kidney and lung are linear (first-order kinetics), metabolic conversion is not a linear function of concentration. Biotransformation by sequential oxidation in the liver accounts for 96.9% of the elimination, while urinary excretion and exhalation account for the remainder (NTP 2002).

2.1.7 2-Methyl-1,2-propanediol and alpha-hydroxyisobutyric acid

It was noted in Section 2.1.3 that in humans and rats, the primary urinary metabolites of MTBE are TBA, 2-methyl-1,2-propanediol (CAS No. 558-43-0), and α-hydroxyisobutyric acid (CAS No. 594-61-6). Values of $t_{1/2}$ ranged from 2.9 to 5 h for urinary excretion of these primary urinary MTBE metabolites in F344 rats, and from 7.8 to 17 h in humans (Amberg et al. 1999)

2.2 Related pharmacokinetic models

Traditional single- or multi-compartment biokinetic models, as well as PBPK models, have been used to estimate a variety of dose-related measures or “metrics” that arise in response to an assumed scenario of administered dosing. Of particular interest are dose metrics that represent biologically effective dose, in the sense that these metrics may best explain (mechanistically) and/or best correlate with a toxicological response of interest. Potentially relevant metrics include peak concentration, average concentration, area under the concentration–time curve (AUC), or total administered dose metabolized either in blood or in specific target tissues. For example, Blancato et al. (2007) and Borghoff et al. (2010) used PBPK models addressing MTBE and TBA to evaluate alternative dose metrics for rats and humans, respectively, exposed to MTBE by different pathways. As explained below, for the purpose of this reassessment of MTBE cancer potency, the brief review and illustration of PBPK models that follows focus on model predictions of the retained fractions of MTBE and of TBA doses that were administered chronically to bioassay rodents in studies of the carcinogenicity of these administered chemicals.

As discussed further in Section 6, MOAs pertaining to environmental chemical carcinogen risk are typically grouped into those with low-dose–response relationships for increased risk that are known or assumed to be either nonlinear (i.e., “sublinear,” upward-curving, threshold-like, or S-shaped) or linear (i.e., LNT). Dose–response relationships that curve upward monotonically with any slope greater than one have potencies that by definition approach zero as dose approaches zero. Consequently, by definition, dose metrics relevant to quantitative assessments of carcinogenic “potency” (i.e., low-dose slope, or increased response per unit dose at very low doses) are those that are consistent with a linear relationship between increased tumor risk and biologically effective dose. Assuming that carcinogenic MOAs are linear (e.g., genotoxic) traditionally has been viewed as a conservative approach to risk assessment for environmental chemical carcinogens—an approach that does not necessarily yield a “best” estimate, but rather one that typically is adopted as a default assumption in the absence of confirming data, intended to avoid underestimating risk so as to err on the side of safety (NRC 1983, 1994; U.S. EPA 1986, 2005a). For environmental (i.e., extremely low-level) scenarios involving chemical carcinogen exposure, zero effective carcinogenic potency is typically assumed to pertain to any proximate, precipitating endpoints that have sublinear MOAs (U.S. EPA 2002, 2005a).

Since (with notable exceptions) most organic chemical carcinogens are “indirect” in the sense that they require metabolic activation to damage DNA, to cause somatic mutations, and to exert a carcinogenic effect, the biologically effective dose metric most relevant to potency assessment is the fraction of daily administered dose that is metabolized to one or more proximate genotoxic forms. As discussed further in Section 6, the focus in this context is typically on lifetime, TWA dose. The specific genotoxic metabolite(s) responsible for tumorigenesis are typically unknown and are merely presumed to exist as a conservative default policy for interpreting a sparse available set of relevant mechanistic data, as an input to regulatory risk analysis and management. However, there is often experimental evidence indicating that the parent, unmethylolated form of an organic chemical carcinogen is not responsible of inducing tumors; a reasonable surrogate measure of biologically effective genotoxic dose often adopted is the (e.g., daily) total metabolized amount of the administered dose. Consequently, for volatile organic chemical carcinogens that in parent (unmethylolated) form are excreted only or nearly exclusively via exhaled breath, a commonly applied surrogate measure of biologically effective dose has been the metabolized (and in this case, non-exhaled or “retained”) portion of administered dose. PBPK models are particularly useful for estimating the retained versus exhaled fractions of administrated volatile chemicals, as well as the disposition of resulting (including any volatile) metabolites. PBPK models can also help explore the consistency of alternative biologically effective dose metrics for cancer and other endpoints.

Section 2.2.1 discusses and illustrates how PBPK models may be applied to estimate retained (i.e., metabolized and non-exhaled) fractions of MTBE doses that were administered to rodents used in bioassays of MTBE carcinogenicity. Section 2.2.2 provides a similar discussion and illustrations pertaining to TBA, noting a current gap insofar as the available PBPK modeling literature has not included any estimates of the total
fraction of any TBA ingested by rats in drinking water that is retained and metabolized rather than exhaled. To address this gap, in Section 2.2.2 we also report estimates of this retained fraction of ingested TBA that we obtained by applying an adaptation of a recent rat PBPK model for TBA in rats. PBPK-derived values of rodent-retained dose presented in Sections 2.2.1–2.2.2 are used later in this reassessment of MTBE carcinogenic potency (Sections 6.1–6.2). Related human PBPK models for MTBE and TBA are mentioned briefly for reference in these two sections, and Section 2.2.3 briefly summarizes PBPK models available for other MTBE metabolites, again only for reference. Predictions of retained MTBE and TBA dose made by human PBPK models are not applied in this reassessment because, as discussed in the introduction to Section 6, this reassessment applied a conservative assumption that 100% of any very small amount of MTBE is metabolized after it is absorbed by any route of exposure.

2.2.1 MTBE

2.2.1.1 Rodent models. Miller et al. (1997) applied compartmental modeling to determine that in F344 rats exposed to MTBE dermally, orally, by inhalation, and by i.v. injection, similar pharmacokinetics were observed for MTBE and TBA administered by the different routes, and the apparent volume of distribution corresponded roughly to body weight (BW), indicating no evident storage compartments for the MTBE or TBA in the rats studied.

PBPK models for MTBE were developed based on experimental data from studies with Fischer 344 (F344) rats (Borghoff et al. 1996, 2010, Rao and Ginsberg 1997, Leavens and Borgoff 2009). The PBPK model developed by Leavens and Borgoff (2009) expanded previously published PBPK models for MTBE to include binding to alpha2u-globulin in the kidney of male rats, differed from the Rao and Ginsberg (1997) model by using different values for certain parameters, and differed from that of Borghoff et al. (1996) (whose model described TBA only in a total water compartment) by modeling TBA in all compartments also modeled for MTBE.

Using the PBPK model developed by Leavens and Borgoff (2009), Borghoff et al. (2010) reported PBPK-based estimates of several dose metrics, including the percentage of administered MTBE that was exhaled, for MTBE doses administered to bioassay rats in three chronic exposure scenarios: (1) MTBE administered by ingestion (at 0.5, 3, 7.5, and 15 mg/mL) in drinking water (as in the cancer bioassay of TBA-exposed F344 rats and B6C3F1 mice reported by NTP 1995 and Cirvello et al. 1995, and in the study of MTBE carcinogenicity in Wistar rats done by Dodd et al. 2013); (2) MTBE administered by gavage in oil four days per week, as in the bioassay of MTBE carcinogenicity in male Sprague-Dawley rats done by Belpoggi et al. (1995, 1998); and (3) MTBE administered by inhalation for six h/day, five days/week (as in the bioassay of MTBE carcinogenicity in F344 rats reported by Bird et al. 1997, and in the cancer bioassay involving in F344 rats exposed to gasoline vapor condensate with or without MTBE reported by Benson et al. 2011). These PBPK-based estimates of retained/metabolized dose are listed in Table 2 of Borghoff et al. (2010). Figures 2 and 3 show the corresponding PBPK model predictions of retained MTBE dose after rat exposure to MTBE in air (with retained MTBE expressed in mg/kg BW per day, or mg/kg/day), and to MTBE in water (with retained MTBE expressed as a percentage of ingested MTBE dose), respectively. These figures show that the predicted amounts of retained MTBE are in each case well approximated by a hyperbolic relationship. For male rats exposed to the lower three concentrations in drinking water (0.5, 3.0, and 7.5 mg/mL) shown in Figure 3, the estimated mean ingested daily doses (±1 SD) were 25 ±11, 140 ±63, and 330 ±139 mg/kg/day, respectively (Dodd et al. 2013), and the corresponding estimates of retained (as noted above, meaning non-exhaled) dose were therefore 8.30, 31.4, and 80.2 mg/kg/day, respectively. After male rats were exposed to 250 or 1000 mg/kg/day of MTBE by gavage in oil, PBPK modeling predicts that approximately 19% and 12% of these doses were retained, or 47.5 and 120 mg/kg/day, respectively (Table 2 in Borghoff et al. 2010).

Although the PBPK model developed by Leavens and Borhoff (2009) incorporates sex-specific parameters governing MTBE concentrations in kidney, which reflect saturable, competitive male-specific binding of MTBE and TBA to alpha2u-globulin, the estimated relationships shown in Figures 2 and 3 pertain to male as well as to female rats. This PBPK model structure implies that, although MTBE and TBA binding to alpha2u-globulin is reversible and tends to slow the clearance of the bound chemicals from the male rat kidney, the modeled alpha2u-globulin-binding kinetics do not affect the distribution of these chemicals in the blood or other tissues (Leavens and Borhoff 2009, Borghoff et al. 2010). Thus while modeled male-specific alpha2u-globulin binding is expected to reduce the rate at which MTBE is metabolized after male rats are exposed to MTBE, the model predicts the same total metabolized fraction of MTBE that is administered to male and female rats.

As pointed out at the beginning of Section 2, if an unidentified genotoxic MTBE metabolite were presumed to induce an observed increase in MTBE-related tumor incidence, daily retained MTBE dose might be used as a surrogate measure of effective, TWA daily metabolized dose for the purpose of dose–response assessment used to assess carcinogenic potency. Such a retained dose metric would under these assumptions have the advantage that it ignores unmetabolized MTBE that is exhaled, and so would thus correspond more closely to the stated assumptions. To use a PBPK-based approach to make such calculations of surrogate effective dose applicable to cancer bioassays involving mice exposed to MTBE by inhalation, a relationship applicable to mice analogous to the one that applies to rats shown in Figure 2 would need to be available. For example, this type of mouse-specific information could be applied to estimate surrogate biologically effective doses for cancer bioassays of inhaled MTBE using CD-1 mice reported by Bird et al. (1997), which are considered in Sections 4 and 6. To develop such PBPK model predictions for mice, the rat PBPK model of Leavens and Borhoff (2009) could be adapted to pertain to mice, and then applied as done for rats by Borghoff et al. (2010) to address a typical rodent bioassay inhalation scenario.

In the absence of mouse PBPK data upon which a mouse PBPK model for MTBE can be validated, such an adaptation for mice would need to rely solely on applying allometric interspecies scaling assumptions to all model parameters. Certain PBPK model parameters, such as tissue:blood parti-
each route of exposure examined. The fits were used to estimate average daily doses of 0, 85, 195, or 420 mg TBA/kg BW, respectively (Cirvello et al. 1995, NTP 1995). In order to express the estimate of TBA potency as increased risk per mg/kg/day of retained (i.e., non-exhaled) TBA rather than per mg/kg/day of TBA consumed, the relationship between TBA mass consumed from drinking water and the corresponding retained (non-exhaled) TBA in bioassay rats must be characterized. A greater potency value will be calculated if TBA dose is expressed in terms of retained rather than administered or consumed TBA, to the extent that < 100% of the administered TBA is retained because some of it is excreted in exhaled breath. PBPK models of TBA in rats can be used to estimate the fraction of ingested TBA that is retained and metabolized.

A PBPK model for MTBE and TBA was developed in male F344 rats (Borghoff et al. 1996). Two different models were developed to describe the dosimetry of TBA in the rat and were tested for their ability to predict TBA blood levels after MTBE exposure. Using a five-compartment model (blood, kidney, liver, muscle, and fat) with the measured partition coefficients, the model was able to predict the accumulation of TBA in blood, but not its clearance. A two-compartment model (total body water and liver) best predicted the accumulation and clearance of TBA in rats exposed to 400 ppm (1,440 mg/m³) of MTBE by inhalation, and to 400 or 40 mg/kg of MTBE by oral administration. The information obtained for TBA using both models suggested that clearance of TBA from the blood of rats after exposure to MTBE involved processes beyond metabolic elimination and that TBA kinetics were dose dependent (NSF International 2003). The human PBPK models developed by Rao and Ginsberg (1997) and the rat PBPK model for MTBE developed by Leavens and Borghoff (2009) discussed in Section 2.2.1 each also include models for TBA in all compartments that also were modeled for MTBE. However, Leavens and Borghoff (2009) applied their model to compare its predictions with experimental rat data obtained only for scenarios in which MTBE or TBA was...
inhaled, and not one in which TBA was ingested in drinking water. To address a TBA-ingestion scenario, we adapted the Leavens and Borghoff (2009) PBPK model for TBA in rats as indicated in Figure 4 legend. Figure 4 shows the resulting predicted relationship between ingested TBA and TBA retained in blood estimated for rats drinking aqueous TBA concentrations of 1.25, 2.5, or 5 mg/mL, assuming that the rats drank 10 evenly distributed times during each 12-hr dark cycle (as assumed by Borghoff et al. 2010 for MTBE exposure in drinking water). Figure 5 shows the corresponding predicted patterns of percentage of ingested TBA that is exhaled (i.e., not retained). The mean retained percentages of TBA at dynamic concentrations of ingested TBA—approximately 29.8%, 46.7%, and 60.7%, respectively—are well approximated by the hyperbolic function shown in Figure 6.

2.2.3 Formaldehyde and methanol

As with other soluble and reactive gases, typical PBPK models that predict steady-state blood concentrations are not useful for predicting formaldehyde dosimetry at this time. As noted previously, inhalation exposure to formaldehyde has not been shown to increase blood formaldehyde levels. Thus, most modeling efforts for formaldehyde have focused on disposition at the site of contact (U.S. EPA 2010). Casanova et al. (1991) developed a PBPK model for predicting levels of formaldehyde-induced DPX observed in rats and rhesus monkeys exposed to formaldehyde, allowing PBPK-based extrapolation of these predictions to humans. Conolly et al. (2003) assigned a more specific role for DPX, treating local DPX concentration as a dose surrogate indicative of intercellular formaldehyde concentration, leading to formaldehyde-induced mutations. In this model, formaldehyde flux to a given region of the nasal lining used as input to PBPK modeling was first obtained by a computational fluid dynamic or CFD model of inhaled formaldehyde in air flowing through a highly realistic mathematical model of the nasal cavity; clearance or repair of DPX was modeled as a first-order process, and rat and rhesus monkey parameters were extrapolated to humans to develop the human model.

PBPK models for methanol have been developed that reflect its uptake, distribution, and metabolism in rodents and humans. The one-compartment, “semi-physiologic” PBPK model developed by Perkins et al. (1995a b) describes methanol disposition in mice and rats exposed by various routes, using a model structure that incorporates only a single metabolic compartment.
that does not reflect respiratory or urinary elimination. Earlier PBPK models developed by Horton et al. (1992) for methanol in rats, monkeys, and humans included more compartments, but did not account for fractional absorption and were validated using relatively small animal data sets (NTP 2002).

3. Genotoxicity of MTBE and its metabolites

Toxicity to genetic material or “genotoxicity” has relevance for assessing a chemical’s potential to cause cancer. Genotoxicity is tested using a number of experimental assays, all of which use relatively high doses, such as applied to isolated, cultured cells (i.e., in vitro) or by examining samples of genetic material in cells of animals after chemical exposure. Positive test results are indicative of a potential for carcinogenicity, although substances testing positive are not necessarily human carcinogens at environmentally relevant doses.

Studies addressing the genotoxic potential of MTBE, its metabolites, and of methanol (which generates the MTBE metabolite formaldehyde) are discussed below and summarized in tables contained in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367. Methanol is included because like MTBE it is metabolized to formaldehyde by a common, saturable, CYP mixed-function oxidase enzyme pathway. A comparison of evidence concerning methanol and MTBE genotoxicity to that concerning formaldehyde genotoxicity is therefore particularly relevant to assessing the question of whether exposure of target cells to formaldehyde per se is, in terms of genotoxic consequences, biologically equivalent to metabolism of chemicals like MTBE and methanol that produce formaldehyde as a metabolite.

3.1 MTBE

The genotoxic potential of MTBE has been studied in many different systems, including reverse mutation systems in bacteria (Salmonella Typhimurium) and yeast cells (Saccharomyces cerevisiae), gene mutation assays, and mammalian cellular systems. The potential of MTBE to cause chromosomal aberrations has also been investigated. Genotoxicity studies of MTBE are summarized in Supplementary Table A-1 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 (in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367), which is organized according to test system type.

Results from studies of the potential for MTBE treatment to cause reverse mutations in bacteria and yeast systems were largely negative (EU 2002). An exception was a study by Williams-Hill et al. (1999) in which a positive result was obtained using Salmonella strain TA102: a “weakly” positive result was obtained when the liver fraction with metabolic capability (S9) was not added to the test, and a “moderately” positive result was obtained when S9 from rat liver was used. The European Chemicals Bureau or ECB noted in their 2002 risk assessment of MTBE that it is of importance that this “weak” response was very low, and the significance of the difference in number of revertants between the controls and those incubations that did not receive metabolic activation (by adding S9) is unclear (EU 2002). In the same experiment, addition of human liver S9 diminished the positive result to levels similar to those observed in the absence of S9 fraction from rats. The addition of the formaldehyde-metabolizing enzyme, FDH, to the system inhibited the observed mutagenicity by 25–30%, indicating that formaldehyde formation in the incubation system as an MTBE metabolite was responsible, at least in part, for a portion of the observed, albeit moderate-to-weak, mutagenic activity. Williams-Hill et al. (1999) concluded that an intact DNA-excision repair system, which the TA102 test strain of Salmonella bacteria possesses, was responsible for the weak-to-moderate positive mutagenic effect. However, in their risk assessment report, the EU (2002) concluded that the small amount of formaldehyde likely produced, which was removed on the introduction of FDH, was likely responsible for the effect. The EU further attributed the weakness of the response to differences in metabolic efficiency between rat and human S9 and suggested that this was similar to results observed by Mackerer et al. (1996) in a mouse lymphoma assay (described in Supplementary Table A-1 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367, Section 5.1). The EU also noted, however, that in strains TA100 and TA104, a positive result was obtained for formaldehyde, but no mutagenic potential was observed from formaldehyde as a product of MTBE metabolism, and concluded that TA102 may possibly be more sensitive than strains TA100 and TA104.

The Williams-Hill et al. (1999) study did not use Good Laboratory Practices (GLP) standards to ensure the quality of its study protocol and conduct (McGregor 2006). When analogous tests of mutagenicity were performed by McGregor et al. (2005) using strain TA102 with and without S9 metabolic activation for MTBE and for TBA, in experiments conducted in two separate laboratories using standard procedures outlined in Organization for Economic Co-operation and Development (OECD) Guideline 471 for chemical testing, only negative results were obtained (Supplementary Table A-1 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367).

Gene mutation assays in mammalian cells have produced mixed results for MTBE depending on the test system. In the mouse lymphoma cell assay in a L5178Y TK-/+ culture test system, MTBE treatment resulted in a dose-dependent mutation frequency in the presence of metabolic activation with rat liver homogenate (ARCO 1980). Cells cultured without metabolic activation, however, did not display a meaningful change in mutation frequency, suggesting that metabolic conversion of MTBE to an active compound was required for the observed increase in mutation frequency (EU 2002). Both TBA and formaldehyde have been suggested as putative genotoxic metabolites of MTBE. The genotoxicity of these two metabolites is discussed in more detail below. Briefly however, Mackerer et al. (1996) exposed mouse lymphoma cells to MTBE in the presence and absence of FDH and its cofactor NAD+, demonstrating that mutation frequency resulting from MTBE treatment did not increase in the presence of FDH; however, in the absence of FDH, mutation frequency was increased approximately 5-fold. In vivo, formaldehyde is rapidly detoxified and shunted to one-carbon metabolism by an enzymatic mechanism that first requires reaction of formaldehyde with sulphydryl groups such as GSH and cysteine.
Since neither GSH nor cysteine was specifically included in the Fisher’s medium (other than in supplemental serum) used by Mackerer et al., detoxification mechanisms normally active in vivo were at least partially compromised in their in vitro tests, indicating that the mutagenicity observed in this study evidently was an artifact of unrealistic features of the culture medium and the metabolic activation system used (McGregor 2006). Two other gene mutation studies of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in Chinese hamster V79 cells were negative (Life Science Research 1989a; Cinelli et al. 1992).

MTBE is metabolized by CYP to formaldehyde, a clearly mutagenic smaller molecule (with the molecular formula HCHO) that can form DPX as discussed below, and also formaldehyde-derived RNA–formaldehyde adducts (RFA). Casanova and Heck (1997) studied the formation of DPX and RFA from MTBE using freshly isolated hepatocytes from female CD-1 mice incubated with MTBE-(O-methyl)[14C]. DPX and RFA were detected, but adduct yields were very small and were independent of the concentration of MTBE in the hepatocyte suspension over a wide concentration range (0.33–6.75 mM) (Figure 7). Similar results were obtained using hepatocytes from male B6C3F1 mice and male F344 rats. Induction of CYP by pretreatment of mice with MTBE prior to isolation of hepatocytes did not measurably increase either DPX or RFA yields. In contrast, there was a marked, concentration-dependent increase in yields of both DPX and RFA when [14C]-formaldehyde was added directly to the medium, suggesting that MTBE-to-formaldehyde metabolism approaches saturation at concentrations below 0.33 mM, and that the rate of this metabolic transformation is slow relative to that of endogenous formaldehyde metabolism (Figure 7).

It is noteworthy that the Casanova and Heck (1997) study was done using overlapping formaldehyde and MTBE concentrations measured in mM units, which give each molecule the same value regardless of molecular weight. When metabolized, each molecule of MTBE directly yields exactly one molecule of formaldehyde. Therefore, the Casanova and Heck (1997) study proved that in vitro exposure to extracellular formaldehyde does not have the same effect on cellular DNA as an equal molar exposure to formaldehyde that is generated endogenously as a product of oxidative MTBE metabolism. Those investigators interpreted their data, shown in Figure 7, to indicate that the rate of intracellular formaldehyde production from MTBE was slow relative to that of formaldehyde oxidation to formate, which subsequently was incorporated into the one-carbon pool and thence into purines, DNA, and RNA, consistent with the MTBE metabolism scheme shown in Figure 1. If so, and if this also occurs in rodents and humans generally in vivo, a plausible explanation is that MTBE metabolism occurs principally in cellular mitochondria that concentrate the antioxidant GSH as well as FDH (ADH3) enzyme (see Sections 2.1.2, 2.1.4, 3.2, 4.2).

Assessment of direct DNA damage following MTBE treatment in vivo produced mixed positive and negative results, despite MTBE displaying no molecular characteristics that would suggest any ability to damage DNA through direct molecular mechanisms. Lee et al. (1998) reported a positive finding in a comet assay performed in rat lymphocytes isolated from rats that had received gavage MTBE treatments at a level of 800 mg/kg/day for 28 consecutive days. This publication appeared only in the form of a Society of Toxicology Meeting Abstract, precluding a careful evaluation of the merits of the underlying study.

Evidence of DNA fragmentation induced by incubation of Rat-1 fibroblasts for 0.5, 4, or 12 h in 0.84 mM (−74 ppm) MTBE or in 0.44 mM (−33 ppm) TBA, the concentrations corresponding to 50% inhibition of 48-h growth in medium containing these respective chemicals, was obtained using the alkaline comet (single-cell gel electrophoresis) assay (Sgambato et al. 2009). Another study (Iavicoli et al. 2002) done by the same group of investigators determined that MTBE concentrations of 0.084 and 0.84 mM during exponential growth reduced cell survival to −75% and −40%, respectively, of that in control cells (Figure 8). At these MTBE concentrations, the corresponding percent of cells undergoing apoptosis was increased −1.8- and 11-fold above control levels, respectively (Iavicoli et al. 2002, Table 4). Apoptosis involves DNA fragmentation that also is detected by comet assay, and apoptotic or necrotic fragmentation of cellular DNA is difficult to distinguish definitively from DNA fragmentation that may be induced directly by chemical exposure, unless careful measures are taken at multiple time points to make such distinctions unambiguously (Brink et al. 2006, Brink 2007). Careful measures of cell viability have been recommended to safeguard against unresolved issues concerning cytotoxicity-related comet assay misinterpretation (EFSA 2012). Only detailed measures of viability at multiple time points can effectively distinguish DNA fragmentation due to cell death from the direct, chemically mediated DNA damage that the comet assay detects (Brink et al. 2006, Brink 2007). Such detailed measures of cell death were rarely included as part of comet assays done to date, and were not included in any comet assays applied to MTBE or to any of its metabolites.
Chen et al. (2008) also reported positive findings for DNA damage in a comet assay using isolated lymphocytes from a single human volunteer that were exposed to 200 μM MTBE. Although cell viability was reported as greater than 95% using Trypan Blue staining, the true extent of cell viability in this study is questionable in view of the fact that Trypan Blue-based measures of cell viability systematically underestimate cell death (Altman et al. 1993, Mascotti et al. 2000). Viability data were not presented in the study, and negative control data (0.5% dimethyl sulfoxide [DMSO]) were not included in the tables reporting DNA damage, making independent comparison with controls impossible. This is complicated by the fact that the authors reported using 0.5% DMSO solvent as a negative control while the test samples themselves were dissolved in 1% DMSO. Although unlikely, this 2-fold increase in solvent concentration could have affected cell viability despite the lack of transparency in cell viability data reporting. We could not assess this possibility independently because negative controls were excluded from the data presented. In consideration of this and in the absence of thorough reporting of cell viability data in the Chen et al. (2008) publication, it is not possible to determine whether the observed positive response in the comet assay was a result of cell death or whether DNA damage occurred via direct reaction of one or more MTBE metabolites with DNA as asserted by the authors.

Yuan et al. (2007) reported positive findings for adduction of MTBE (and TBA) to DNA using accelerator mass spectrometry (AMS) methods, but the technique used and its corresponding method of application were and remain incapable of determining whether radiolabeled DNA detected is the result of adduct formation, or simply the result of endogenous carbon metabolism and recycling resulting in the incorporation of labeled carbon into nucleotide pools used to synthesize and/or repair DNA via processes that are unrelated to adduct formation (Wang et al. 2004, McGregor 2010, Zhu et al. 2010). Another study reporting very similar positive results from the same research group, Du et al. (2005), also suffers from this same flaw in that it failed to verify that the formation of AMS-detected radioisotope was the result of direct covalent binding of an MTBE metabolite to DNA. Finally, Tang et al. (1997) reported DNA strand breakage in human HL-60 leukemia cells following MTBE treatment, but the publication was available only in Chinese.

Assays of unscheduled DNA synthesis (UDS) were negative for MTBE, except for one positive assay in rat primary hepatocytes (Zhou et al. 2000). The authors concluded that the positive finding may indicate “some degree of mutagenicity” for MTBE; however, some including McGregor (2006) critically reviewed this paper and concluded that the method used by Zhou et al. (2000) to assess UDS, scintillation counting, has been considered invalid for many years. Furthermore, the other two existing studies of UDS in rat primary hepatocytes resulting from MTBE treatment are both negative (Life Science Research 1989b; Cinelli et al. 1992).

Cytogenetic assays performed with MTBE in vitro were all negative or equivocal, including sister chromatid exchange (SCE) assays, chromosomal aberrations analysis, and a mouse micronucleus (MN) test. Cytogenetic assays performed in vivo, including a sex-linked recessive lethal test in Drosophila melanogaster, UDS in mouse hepatocytes, HPRT gene mutation in spleen lymphocytes, two mouse micronulei tests, a bone marrow cytogenetic test, and an assay for chromosomal aberrations in the bone marrow of male Sprague-Dawley rats were all negative.

A cell transformation assay using C3 H 10T1/2 C18 mouse embryo fibroblasts, treated for 24 h at 0.336- or 0.672-nM concentrations of MTBE, resulted in 2.1- and 2.5-fold (equivocally) increased rates of cell transformation, respectively (Iavicoli et al. 2002). These results conflict with a negative earlier finding from the same laboratory (Litton Bionetics Inc., 1980), as noted in Table 4. The significance of the finding by Iavicoli et al. (2002) in relation to a hypothetical genotoxic MOA is questionable in view of cytotoxicity that very likely was induced at the tested concentrations (Figure 8), and this alone may have indirectly induced the observed increase in transformation rates. Concentrations selected for the cell transformation assay were reported not to produce cytotoxicity in preliminary tests after 24 h of treatment (data not shown). However, these concentrations clearly could substantially reduce cell survival when measured at 48 h (Figure 8), and some delay in the expression of chemically induced cell death is generally expected (Brink 2007). The demonstrated cytotoxicity of test concentrations used when evaluated at 48 h, and a lack of more detailed survival data, make it plausible that commitment to impending cell death may have occurred at MTBE concentrations that caused cell transformation in Rat-1 fibroblasts. Specifically, positive results in the comet assays and oxidative DNA damage test by Sgambato et al. (2009) listed in Supplementary Table A-1 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 clearly

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**Figure 8.** Reduced growth of normal rat Rat-1 fibroblasts incubated for 48 h with different MTBE concentrations, as determined by colorimetric assay quantifying the formazan-dye metabolite generated only in living cells that are incubated with its precursor, MTT (each point = mean percent of control by three independent experiments, error bars denote ± 1 SD). Adapted from Figure 1 of Iavicoli et al. (2002).
showed that relatively high MTBE concentrations can induce oxidative stress. Oxidative stress, in turn, shifts the “redox state” (i.e., balance between reduced vs. oxidized molecules) in cells toward oxidation levels that can trigger and mediate an apoptotic signaling pathway that leads to programmed cell death (Buttke and Sandstrom 1994, Haddad 2004). Likewise, oxidative stress per se can induce positive results in a cell transformation assay (Zhang et al. 2000).

In summary, although in vitro cell transformation assays may be included together with a battery of in vitro genotoxicity screening tests to assess for potential carcinogenicity, results from cell transformation assays—like those comet assays as discussed above—can be misleading if the magnitude of exposure-related cell killing is not determined at multiple time points. Lack of detailed cell survival data in this study thus renders the biological significance of the observed transformation assay results impossible to interpret meaningfully. Thus, the overall evidence for MTBE indicates that it is unlikely to be genotoxic.

### 3.2 Formaldehyde

Formaldehyde genotoxicity has been studied extensively. Formaldehyde is positive in mutagenic assays, in assays for DNA and protein adduct and crosslink formation, and in assessments of chromosomal aberrations, aneuploidy, and DNA breakage. Overall, the ability of formaldehyde to produce positive results in a variety of genotoxicity assays is not disputed, but the relevance of formaldehyde treatment tested in mutagenicity and cyogenetic test systems to the genotoxic potential of formaldehyde in the context of MTBE metabolism has been debated. The presence of effective and efficient formaldehyde detoxification systems in vivo, namely the metabolic enzyme, FDH, appears to provide protection against the genotoxic effects of formaldehyde in the context of MTBE metabolism.

Formaldehyde is inherently reactive and is capable of interacting with several biomolecules, including DNA and proteins (U.S. EPA 2010). Several reports of DNA–protein crosslinks are presented in Supplementary Table A-2 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 (in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367), as well as DNA–DNA crosslinks and DNA adducts. Complicating this, formaldehyde is also formed endogenously (in the body). In its 2010 assessment of formaldehyde in support of Integrated Risk Information System (IRIS), EPA cautioned that formaldehyde reaction products are difficult to quantify, because many of the products formed in vivo are labile (Quievryn and Zhitkovich 2000) and difficult to measure (U.S. EPA 2010) and that, furthermore, formaldehyde is metabolically incorporated into nucleic acids. As a result, presumed adducts of formaldehyde with nucleic acid must be interpreted carefully to distinguish between covalently bound and metabolically incorporated formaldehyde (Fennell 1994, Casanova et al. 1989, Casanova and Heck 1987, Casanova-Schmitz et al. 1984a, b, Casanova-Schmitz and Heck 1983). Endogenous tissue formaldehyde concentrations are similar to (~0.05-mM) concentrations that induce genotoxicity and cytolethality in vitro (Heck and Casanova 2004). Heck and Casanova reviewed evidence indicating that inhalation of formaldehyde failed to generate (1) any detectably increased formaldehyde blood level in rats, monkeys, or humans exposed to concentrations of 14.4, 6, or 1.9 ppm, respectively; (2) detectable formaldehyde–protein adducts (FPA) or DPX in the bone marrow of normal rats exposed to up to 15 ppm of [3 H]- and [14C]formaldehyde; (3) detectable FPA or DPX in the bone marrow of glutathione-depleted rats exposed to up to 10 ppm [3H]- and [14C]formaldehyde; (4) detectable DPX in the bone marrow of rhesus monkeys exposed to up to 6 ppm of [14C]-formaldehyde; and (5) chromosomal aberrations in bone marrow of rats exposed to airborne formaldehyde concentrations up to 15 ppm or of mice injected i.p. with formaldehyde doses up to 25 mg/kg.

The few positive genotoxicity assays for MTBE have been attributed in large part to metabolism of MTBE to formaldehyde or other metabolites, rather than to MTBE itself, in accordance with observations that MTBE must be metabolized to formaldehyde in order to be genotoxic, and that further metabolic systems exist (FDH) for effectively metabolizing the formaldehyde generated to relatively more non-toxic species (Conaway et al. 1996). As discussed briefly above, Mackerer et al. (1996) demonstrated that the addition of FDH to a mouse lymphoma cell gene mutation assay was able to decrease mutation frequency in the presence of an exogenous metabolic system to levels similar to those observed when no metabolic system was present. Casanova and Heck (1997) also showed that CD-1 mouse hepatocytes exposed to up to mM concentrations of formaldehyde exhibit a striking accumulation of DPX in direct proportion to MTBE concentration, whereas exposure instead to up to 7-mM concentrations of MTBE yields no discernible increase in DPX above background levels at any concentration tested (see Figure 7, Section 3.1). As noted in Section 3.1, this study thus showed that exposure to extracellular formaldehyde does not have the same effect on DNA as an equal exposure to formaldehyde generated endogenously from oxidative MTBE metabolism, due likely to the fact that MTBE metabolism occurs by oxidative enzymes located principally in mitochondria that also store the antioxidant GSH as well as the enzyme FDH (ADH3), which both normally act to detoxify endogenous formaldehyde. The EU (2002) risk assessment report on MTBE noted that the positive result from the Mackerer et al. (1996) study is not relevant because it involved MTBE metabolized to formaldehyde outside the cell in an artificial metabolic environment, whereas formaldehyde generated in vivo by MTBE metabolism adds to formaldehyde that normally is generated at intracellular sites of oxidative metabolism.

The potential biological relevance of “exogenous” formaldehyde–DNA adducts—those formed by reaction between DNA and formaldehyde arising either from external exposure to that chemical, or to a chemical that becomes metabolized to formaldehyde—can be understood only in view of their magnitude relative to “endogenous” formaldehyde–DNA adducts that are formed when DNA reacts with formaldehyde normally present in the body. In the nasal epithelium of rats exposed to formaldehyde by inhalation, formaldehyde–DNA adducts are formed with a pronounced nonlinear dose–response pattern (Monticello et al. 1996, Lu et al. 2010, 2011). Figure 9 shows fits obtained to data on formaldehyde concentration dependence of exogenous N²-hydroxymethyl-dG adducts
detected by Lu et al. (2010, 2011) in male F344 rat nasal epithelium DNA after 6 h of formaldehyde exposure in vivo by inhalation. Over the same concentration range, the number of endogenous dG adducts did not change significantly (Lu et al. 2010, 2011). Figure 10 shows fits to the corresponding data from the same study obtained for the ratio of exogenous to endogenous dG adducts detected in nasal epithelium DNA of the same formaldehyde-exposed rats. Although the dose–response relationships shown in Figures 9 and 10 are each highly nonlinear overall, both are nevertheless consistent with a linear dose–response relationship at the very lowest air concentrations of formaldehyde investigated. These data clearly indicate, however, that at relatively high (> 5-ppm) air concentrations of formaldehyde, mechanisms that normally serve to limit formaldehyde–DNA adducts in rat nasal epithelial cells become saturated and increasingly less effective at increasing formaldehyde concentrations. This nonlinear pattern of formaldehyde–DNA adduct formation in relation to formaldehyde concentrations to which rat nasal tissue was exposed parallel the concentration–response pattern observed for formaldehyde-induced rat nasal tumors (discussed in Section 4.2).

Lu et al. (2010) also compared formaldehyde-induced dG adducts, deoxyadenine (dA) adducts and dG–dG crosslinks in the nasal epithelium of rats exposed by inhalation to 10 ppm of formaldehyde in air for one or five days (6 h/day) to adduct loads detected in six other tissues (lung, liver spleen bone marrow, thymus, and blood), using adduct-specific detection limits of ∼240, ∼75, and ∼60 amol, respectively. Exogenous adducts of any kind were detected only in nasal tissue, the predominant site of contact resulting in formaldehyde uptake from air in rats exposed by inhalation. In nasal tissue, no exogenous dA adducts were detected, and among exogenous dG adducts and dG–dG crosslinks detected, the ratio of the former to the latter was ∼10 after either one or five days of exposure (Lu et al. 2010). Detected levels of exogenous dG adducts and dGdG crosslinks in nasal tissue each increased ∼2-fold after five days of exposure from those detected after only 1 day of exposure, and the five-day exposure period resulted in exogenous dG adduct levels ∼15% less than the detected levels of endogenous dG adducts (Lu et al. 2010). This study clearly showed that up to five days of respiratory exposure to 10 ppm of formaldehyde for 6 h/day did not result in any detectable increases above background levels of DNA adducts or cross-links in any extra-nasal tissues of exposed male F344 rats.

Moeller et al. (2011) use ultra-performance liquid chromatography and mass spectrometry to measure endogenous and exogenous N2-hydroxymethyl-dG adducts, with a 20-amol detection limit, in nasal epithelium and bone marrow of Cynomolgus macaques following two consecutive days of whole-body inhalation exposure to air concentrations of 1.9 or 6.1 ppm of 13CD2-formaldehyde. Endogenous N2-hydroxymethyl-dG adducts were detected in the nasal DNA of all animals studied at an average (±1 SD) of 2.24 ± 0.50 adducts/107 dG, and such endogenous adducts were detected at 17.5 ± 2.6 and 12.4 ± 3.6 adducts/107 dG in bone marrow DNA from the 1.9- and 6.1-ppm exposures, respectively. However, no exogenous adducts were detected in bone marrow, from which ∼10-fold greater amounts of DNA were analyzed, indicating that the formaldehyde exposures studied were not able to increase DNA adducts in bone marrow detectably above the background detected level of such adducts.

Formaldehyde was observed to be unable to induce aneuploidy in cultured V79 cells and in freshly cultured human mononuclear lymphocytes (Speit et al. 2011a) and similar assays (Supplementary Appendix A-2 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). A key question concerning formaldehyde genotoxicity and possible tumorigenic MOAs is whether exogenous formaldehyde may exert systemic genotoxic effects in vivo. Speit et al. (2009) examined blood sampled from male F344 rats after they had inhaled concentrations 0, 0.5, 1, 2, 6, 10, or 15 ppm formaldehyde for 4 weeks (6 h/day, 5 days/week), for evidence of systemic genotoxic effects measured using the tail-moment and tail-migration versions of the comet assay (both without and with 2 Gy of gamma irradiation, to add sensitivity to detect DNA–protein crosslinks), the SCE test, and the peripheral blood MN test. Formaldehyde exposure did not induce any significant effect in any of the systemic genotoxicity tests

![Figure 9](image-url) - Data (open points) relating exogenous dG adducts (on the vertical or Y axis) detected by Lu et al. (2011) in rat nasal DNA after a 6-h in vivo exposure, to different formaldehyde concentrations (on the horizontal or X axis). Error bars = ± 1 SE. Inverse-variance-weighted least-squares nonlinear regression fits shown are $Y = 0.095 X + 0.000183 X^2$ (solid curve); $Y = 150 \Phi((X-27.0)/8.20)$ (dashed curve), where $\Phi$ is the standard normal cumulative probability distribution function. Both fits are very accurate ($R^2 = 0.998$).

![Figure 10](image-url) - Data (open points) relating the ratio of exogenous to endogenous dG adducts (Y axis) detected by Lu et al. (2011) in rat nasal DNA after a 6-h in vivo exposure, to different formaldehyde concentrations (X axis). Error bars = ± 1 SE. Inverse-variance-weighted least-squares nonlinear regression fit shown is $Y = 0.0246 X + 0.0000383 X^4$ (solid curve, $R^2 = 0.999$).
examined, while positive results were detected using blood from rats treated either with 50 mg/kg of methyl methanesulfonate by gavage 4 h prior to sampling (four versions of the comet assay, and the SCE test), or treated orally with 10 mg/kg of cyclophosphamide (CPP) on two consecutive days followed 24 h later by blood sampling (the MN test). Speit et al. (2011b) subsequently measured MN frequency in the nasal epithelium of male F344 rats exposed as in their 2009 systemic genotoxicity study summarized above. They did not detect increased MN frequencies either in any exposed group relative to that in the control rats, or in rats administered a single 20-mg/kg dose of CPP by gavage and analyzed 3, 7, 14, or 28 days later. Kleinnijehuis et al. (2013) investigated the ability of formaldehyde to move from its site of contact to distal body regions where it might exert genotoxic effects, by measuring formaldehyde concentrations in blood after rats inhaled 10 ppm of \( ^{13} \text{C} \)-radiolabeled formaldehyde in air for 6 h. Since this study did not detect elevated radiolabel in blood either during or up to 30 min after the inhalation exposures, using a method sufficiently sensitive to detect as little as a 1.5% increase over background formaldehyde concentrations in rat blood, this study added to previous observations (e.g., Heck and Casanova 2004, Speit et al. 2009) that exogenous formaldehyde is not detectably transported systemically and so cannot induce systemic genotoxic or other effects.

Some other studies, however, have reported potentially genotoxic and cytotoxic effects of systemically distributed formaldehyde. Significantly positive comet assay results were reported for lung cells sampled from formaldehyde-exposed specific-pathogen-free male Sprague-Dawley rats that were exposed 6 h/day, 5 days/week, for two weeks to 0, 5, or 10 ppm formaldehyde (Sul et al. 2007). Ye et al. (2013) measured DPX—as well as GSH, reactive oxygen species (ROS), and malondialdehyde (MDA, a marker for oxidative stress)—in lung, peripheral blood mononuclear cells (PBMC), bone marrow, liver, spleen, and testes of male BALB/c mice that were via nose-only inhalation exposed 8 h/day for 7 consecutive days to airborne formaldehyde concentrations of 0, 0.5, 1.0, or 3.0 mg/m\(^3\) (0, 0.41, 0.81, or 2.4 ppm). Highly significant dose-related trends were observed for DPX, ROS, and MDA in all tissues (positive trends, except no trend for lung DPX), and for GSH depletion in all tissues (negative trends), Supplemental oral administration of 100 mg of GSH/kg/day to mice via intra gastric gavage, 10 min after the end of exposure for a second group of mice otherwise similarly exposed to 3.0 mg/m\(^3\) formaldehyde was observed to increase GSH levels slightly, and to reduce DPX, ROS, and MDA measures in all tissues (except for GSH in lung), compared with non-GSH supplemented mice exposed to 3.0 mg/m\(^3\) of formaldehyde.

Results from several recent studies continue to address the plausibility of the hypothesis that exogenous formaldehyde can induce cytotoxic and genotoxic effects at internal sites after systemic circulation. Zhang et al. (2010) reported that formaldehyde is potently cytotoxic to circulating hematomal precursor cells, and that a comparison of myeloid progenitor cells cultured from blood samples taken from Chinese workers exposed versus controls not occupationally exposed to formaldehyde indicated exposure-related aneuploidy characteristic of myeloid leukemia and myelodysplastic syndromes. Based on a reanalysis of raw data from that study, Gentry et al. (2013) concluded that that differences in blood parameters measured by Zhanget al. (2010) are not clinically or biologically significant, that no in vivo metaphases in workers’ blood cells had been examined in that study, and that evidence of aneuploidy observed in that study was likely to have arisen late during experimental in vitro culture rather than in vivo prior to experimental culture. More recently, Ji et al. (2014) examined MN frequency in C57BL/6 J mouse erythropoietic bone marrow cells (EMC) cultured for 24 h in 0, 25, 50, 75, or 100 \( \mu \)M formaldehyde, and in human PBMC-derived erythroid progenitor cells (EPC) cultured for 10 days in initial concentrations of 0, 25, 50, 100, or 150 \( \mu \)M formaldehyde. An unequivocally (and approximately linearly) increased MN frequency was observed in relation to formaldehyde concentration to which mouse EMC were exposed, and expansion of human EPC declined unequivocally at all but the lowest formaldehyde concentration examined. The frequency of certain aneuploidies involving chromosomes 7 and/or 8 in EPC was also significantly elevated for cells exposed to 50 but not to 100 \( \mu \)M formaldehyde. The most recent study bearing on the potential for systemic genotoxic effects due to exogenous formaldehyde is the report by Yu et al. (2014) that bone marrow cytotoxicity was induced via oxidative stress in juvenile male ICR mice exposed for 15 days to airborne formaldehyde concentrations of 0, 20, 40, or 80 mg/m\(^3\) (0, 16, 32.5, or 65 ppm). Bone marrow cell density was slightly decreased in all exposed groups, significantly so in the highest exposure group. Multiple measures indicated that oxidative stress was elevated in bone marrow cells in all or in one or of the highest exposure groups, and S-phase arrest of these cells was significantly elevated in the highest exposure group.

### 3.3 TBA

Genotoxicity and mutagenicity studies for TBA are summarized in Supplementary Table A-3 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 (in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Overall, results of the studies indicate that TBA is neither genotoxic nor mutagenic. Results of those assays that were positive either were not confirmed in independent studies (McGregor 2010) or possessed flaws that precluded meaningful interpretation of the assays. Other than the unsubstantiated study by Williams-Hill et al. (1999), adequate evidence does not exist to support a conclusion of genotoxicity in reverse mutation studies for TBA.

In an assay of reverse mutation in the Salmonella Typhimurium strain TA102, TBA treatment resulted in a positive response (Williams-Hill et al. 1999); however, in two additional studies conducted under GLP standards, no significant increases in mutation frequency were observed using either DMSO or water as vehicles in the same strain (McGregor et al. 2005). Additional assays of reverse mutation in other strains of Salmonella Typhimurium are also negative (Zeiger et al. 1987). EG&G Mason (1981a) observed a positive result with and without metabolic activation in TA1535; however, Zeiger et al. (1987) failed to confirm this, and further studies with the TA1535-derived and more sensitive strain TA100 were also negative (Zeiger et al. 1987, EG&G 1981b).
Several publications that evaluated MTBE genotoxicity also evaluated TBA genotoxicity by similar or identical methods. Sgambaro et al. (2009) reported that DNA fragmentation was increased as measured by the single-cell gel electrophoresis (or “comet”) assay following TBA treatment; however, as with the MTBE data described above, neither viability nor positive-control data were included in the publication for comparison, so the authors’ conclusion that a purely genotoxic mechanism of damage occurred cannot be verified or adequately assessed. Yuan et al. (2007) reported the formation of TBA-induced DNA adducts concurrent with their report of MTBE adducts, but their method of adduct detection cannot differentiate between true adducts and carbon metabolically incorporated through natural processes into the DNA structure. The results of Tang et al. (1997) are also questionable, in particular because the cell system used in the assay, HL-60 cells, does not possess inherent metabolic capacity; based on results of genotoxicity studies using MTBE alone, metabolic capacity is required to convert nongenotoxic MTBE to genotoxic metabolites in order to yield positive results in genotoxicity assays (McGregor 2010).

Other positive results in genotoxicity assays for TBA include a SCE assay conducted by NTP (1995) considered to be only weakly positive that was not corroborated in a replicate NTP assay, and a single finding of increased mutation in mouse lymphoma L5178Y/TK cells both with and without exogenous metabolic activation (McGregor et al. 1988). In the mouse lymphoma test system, the results indicated weak mutagenicity, with a small but significant increase relative to solvent control at the highest concentrations observed both with and without metabolic activation. Based on this, the authors concluded that TBA demonstrated no evidence of mutagenicity. In agreement with the finding of no mutagenic potential for TBA, EG&G (1981c) reported, in a similar test system with and without metabolic activation, that TBA was negative for mutagenicity.

### 3.4 Methanol

A thorough IPCS review of the genotoxicity studies of methanol concluded that the structure of methanol, by analogy with ethanol, does not suggest that it would be genotoxic (WHO IPCS 1997). Indeed, the majority of studies of genotoxicity and mutagenicity of methanol are negative, although some cytogenetic assays conducted after administering a relatively high dose (1 g/kg) in vivo were positive. Bioassays of methanol overall, however, include some evidence indicating that methanol is a carcinogen, as discussed further in the section on methanol chronic/carcinogenicity studies (Section 4.4). Studies of the mutagenic and genotoxic potential of methanol are summarized in Supplementary Table A-4 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 (in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367).

Cytogenetic and other assays that were positive following methanol treatment include the assays of Pereira et al. (1982), wherein hemoglobin binding, aneuploidy, SCE, and micronuclei formation were all detected as a result of methanol treatment. Crebells et al. (1989) also observed chromosomal malsegregation in Aspergillus nidulans, and Chang et al. (1983a) observed “some” positive evidence in the bone marrow cytogenetic assay in mice following methanol treatment. Lu et al. (2012) observed the formation of hydroxymethyl DNA adducts following methanol treatment, but using a carefully designed radiolabeled treatment approach they demonstrated that the detected adducts formed were due to endogenous formaldehyde and not to the administered methanol. This study result therefore cannot be considered positive.

In the only study reporting positive mutagenic effects, McGregor et al. (1985) observed an increased mutation frequency in a mouse lymphoma cell model for gene mutation in the presence of exogenous metabolic activation but not without exogenous metabolic activation. Additionally in this study, under conditions that increased mutation frequency, the relative total growth, a metric used to demonstrate cell viability, was decreased to greater than 30%, indicating significant toxicity to the treated cells. The significant toxicity concurrent with mutation complicates data interpretation and makes the significance of this result questionable.

As stated above, overall, evidence for methanol as a mutagen does not exist, and evidence of cytogenetic activity is mixed.

### 3.5 Formic acid

Studies of the genotoxicity of formic acid overall indicate that formic acid is not inherently genotoxic or mutagenic, but that when applied to systems directly without buffer as a strong acid, it exerts effects due to its acidity and not due to inherently genotoxic or mutagenic properties. Studies of the mutagenic and genotoxic potential of formic acid are summarized in Supplementary Table A-5 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 (in Supplementary Appendix A to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367).

In an assay of mutagenicity performed in Escherichia coli, Demerec et al. (1951) observed positive results for mutagenicity of formic acid; however, the incidence rate of mutation was not dose dependent. Buffered solutions of formic acid were tested by the NTP (1992) in similar assays but using different strains of E. coli, and these assays were negative or equivocal. Similar to this, Stumm-Tegeloff (1969) observed positive results in a Drosophila sex-linked dominant lethal assay; however, on neutralization, the results were negative, indicating that the acidic property of unbuffered formic acid was the only reason for the positive result.

Sipi et al. (1992) observed a slight but statistically significant increase in SCEs following formic acid treatment; however, the authors strongly advise that this result should be interpreted with caution, because decreased cell proliferation was also indicated for concentrations of formic acid that produced increased SCEs. Assay conditions that only produce genotoxic effects at concentrations that also produce significant cytotoxicity are of questionable relevance to genotoxicity assessment.

Finally, Wang et al. (2004) reported the only other positive result for genotoxicity following treatment with formic acid not due to acidity per se. As with publications previously described for MTBE and TBA, the authors claim to have used AMS to
detect adducts to DNA, presumably resulting from treatment with radiolabeled formate—the conjugate ion of formic acid. However, AMS by itself is a method that simply counts atomic radioisotope ratios (in this case, it counts carbon atoms that differ slightly in atomic weight), and therefore it is incapable of discriminating between actual adducts to DNA resulting from chemical modification of DNA structures and carbon simply incorporated into DNA by natural carbon recycling mechanisms in the body, unless the application of AMS is coupled with mass-spectrometry-based confirmation of putative DNA adducts (McGregor 2010, Zhu et al. 2010), which was not the case in the study by Wang et al. (2004). Normally present in cells, formate (like serine and glycine) is used as a methylene donor during normal cellular metabolism, including the synthesis of nucleotides used to manufacture DNA. The positive result for adducts reported in that study is therefore speculative, unconfirmed, and likely to be erroneous.

### 3.6 Alpha-hydroxyisobutyric acid

Only one study of the genotoxic or mutagenic potential of α-hydroxyisobutyric acid was located. The study was not available in English, but the abstract is available and the study has been reviewed by McGregor (2010) and others. In this study by Tang et al. (1997), increased DNA damage following alpha-hydroxyisobutyric acid treatment was detected by the “comet assay” (single-cell gel electrophoresis), and cytotoxicity was assessed by lactate dehydrogenase measurement. At concentrations that did not induce cellular toxicity, Tang et al. reported that similar levels of DNA damage were detected following treatment with equal concentrations of MTBE, TBA, formaldehyde, or alpha-hydroxyisobutyric acid. McGregor (2010) suggest that there are problems with this conclusion, considering the test method. In particular, it is highly unlikely that all of the compounds tested exerted genotoxic effects within the same small concentration range of 1–30 mM. If all chemicals tested were to have a common metabolite, a scenario of similar mutagenic potency could be possible for each of the four chemicals tested; however, no exogenous metabolic system was added to HL-60-cell test system used, and those cells lack the metabolic potential required to transform all chemicals tested to such a hypothetical common metabolite. The results of this entire study are therefore questionable.

### 3.7 2-Methyl-1,2-propanediol

No genotoxicity or mutagenicity data were located for the MTBE metabolite 2-methyl-1,2-propanediol.

### 3.8 Summary of genotoxicity of MTBE and its metabolites

Table 1 presents an overall categorical evaluation of results of published genotoxicity tests on MTBE, on MTBE metabolites, and on the precursor methanol of the MTBE metabolite formaldehyde, which are summarized individually in Supplementary Appendix A (Tables A-1–A-5) to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367. For MTBE there is not a single unequivocally positive test result from a total of 53 tests performed, including in vivo studies and studies performed with and without metabolic activation in vitro, using assumptions and interpretations noted in Table 1. The data summarized in Table 1 therefore provide strong evidence for lack of genotoxic potential for MTBE administered in vivo at doses that do not induce substantially increased rates of dose-induced cell death.

The chemicals methanol (with one unequivocally positive test result from a total of 34 tests performed), formic acid (or formate, with one reported positive test result from a total of 17 tests performed), and TBA (with no unequivocally positive test results from a total of 25 tests performed) likewise generated a great preponderance of negative genotoxicity test results. The single positive result for formic acid was from a bacterial assay conducted in 1951, before modern standardized reverse mutation test protocols were developed. So all evidence or the great preponderance of evidence indicates that the MTBE metabolites TBA and formic acid are not genotoxic chemicals, and this is also true for the chemical methanol that like MTBE generates formaldehyde as a metabolite in the body.

The pattern of results for formaldehyde is remarkably different. Genotoxicity tests of this MTBE metabolite produced a total of 226 unequivocally positive test results out of a total of 308 tests performed—a positivity rate of 73.4%, using assumptions and interpretations noted in Supplementary Table A-2 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367. At externally administered concentrations that overwhelm defense mechanisms that normally operate in mammalian cells to limit endogenous formaldehyde concentrations, exogenous formaldehyde clearly can damage DNA and cause mutations, as well as kill cells. It is highly noteworthy, therefore, that when MTBE is administered to mammals in vivo—after which each retained (metabolized) MTBE molecule creates one molecule formaldehyde along with one molecule of TBA—a negative overall pattern of genotoxicity occurs. This striking difference in the pattern of genotoxicity test results indicates a fundamental, unequivocal difference between exogenously administered formaldehyde, and formaldehyde generated endogenously by P450-mediated oxidative metabolism of chemicals like MTBE and methanol. Formaldehyde endogenously generated by this pathway from these chemicals is evidently quite efficiently neutralized by intracellular GSH and/or other antioxidants at rates faster than the DNA-reactive formaldehyde metabolite can be generated by the saturable oxidative metabolism that initiates conversion of MTBE to its metabolites.

### 4. Tumorigenicity of MTBE and its metabolites

Data on tumorigenicity of MTBE, MTBE metabolites, and on the precursor methanol of the MTBE metabolite formaldehyde are reviewed below, and summarized in Supplementary Appendix B to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367.

#### 4.1 MTBE

Several studies of the chronic effects of MTBE treatment have been conducted, and neoplastic lesions have been observed in some of these studies. These studies are summarized in Supplementary Table B-1 (in Supplementary Appendix B to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367), and are discussed below.
### Table 1. Summary of MTBE and MTBE-metabolite-related genotoxicity.

| Chemical / Test type | With exogenous metabolic activation | Without exogenous metabolic activation or without specification of metabolic activation | Notes |
|----------------------|-------------------------------------|------------------------------------------------------------------------------------|-------|
|                      | Pos.  | Neg.  | Equiv.* | Pos.  | Neg.  | Equiv.* | “+” and “+/-” findings in TA102 by William-Hill et al. 1999 conflict with those by McGregor et al. 2005 and RBM 1996. |       |
| Reverse or other mutation | 0/13  | 10/13 | 3a+/s/13 | 0/14  | 13/14 | 1b/14 |       |
| DNA adducts or other damage | ND ND ND | 0/8  | 2/8  | 6a+/b/8 |       |
| Unscheduled DNA synthesis | 0/21  | 2/1  | 0/1 | 0/4  | 3b/4 | 1a/4 |       |
| Cyto genetic damage | 0/3  | 1/3 | 0/3 | 0/10 | 9/10 | 3b/10 | One “+” result was in yeast cells at a cytotoxic concentration; 4 “+” results each involved a 1-g/kg dose to mice, which exceeds the mouse LD50. | Notes |
| Chemical: Formaldehyde | With exogenous metabolic activation | Without exogenous metabolic activation or without specification of metabolic activation | Notes |
| Test type / Reverse or other mutation | Pos.  | Neg.  | Equiv.* | Pos.  | Neg.  | Equiv.* | “+” findings at cytotoxic concentrations used by ARCO 1980 and Mackerer et al. 1996, evidently (from data in the latter study) due to accumulation of formaldehyde in the culture medium to concentrations unlikely to occur in vivo. |       |
| Reverse or other mutation | 23/34 | 8/34 | 3/34 | 50/83 | 27/83 | 6/83 |       |
| DNA-protein crosslink forma tion | 0/0 | 0/0 | 0/0 | 58/61 | 2/61 | 1/61 |       |
| DNA adducts or crosslinks | 0/0 | 0/0 | 0/0 | 22/24 | 1/24 | 2/24 |       |
| DNA strand breaks or repair inhibition | 0/0 | 0/0 | 0/0 | 22/29 | 5/28 | 1a+/2/7 |       |
| Unscheduled DNA synthesis | 0/0 | 0/0 | 0/0 | 5/6 | 1/6 | 0/6 |       |
| Cyto genetic damage | 5/5 | 0/5 | 0/5 | 41/69 | 12/69 | 1/69 |       |
| Chemical: t-Butanol (TBA) | With exogenous metabolic activation | Without exogenous metabolic activation or without specification of metabolic activation | Notes |
| Test type / Reverse or other mutation | Pos.  | Neg.  | Equiv.* | Pos.  | Neg.  | Equiv.* | “+” findings of NTP 1995 or “+/-” finding of EG&G Mason 1981d conflict with negative findings by NTP 1995. |       |
| Reverse or other mutation | 0/8 | 6/8 | 2b/8 | 0/9 | 6/9 | 3b+/y/9 |       |
| DNA adducts or other damage | 0/0 | 0/0 | 0/0 | 0/3 | 0/3 | 3a+/3/3 |       |
| Cyto genetic damage | 0/4 | 4/4 | 0/4 | 0/5 | 3/5 | 2b+/5 |       |
| Chemical: Methanol | With exogenous metabolic activation | Without exogenous metabolic activation or without specification of metabolic activation | Notes |
| Test type / Reverse or other mutation | Pos.  | Neg.  | Equiv.* | Pos.  | Neg.  | Equiv.* | “+” findings of Pereira et al. 1982 not considered a direct demonstration of genotoxicity. |       |
| Reverse or other mutation | 1/5 | 4/5 | 0/5 | 0/9 | 9/9 | 0/9 |       |
| Protein binding | 0/0 | 0/0 | 0/0 | 0/1 | 0/1 | 1b/1 |       |
| DNA adducts or other damage | 0/0 | 0/0 | 0/0 | 0/4 | 4 | 1b/4 |       |
| Cyto genetic damage | 0/0 | 0/0 | 0/0 | 0/15 | 10/15 | 5b/15 |       |
| Chemical: Formaldehyde | With exogenous metabolic activation | Without exogenous metabolic activation or without specification of metabolic activation | Notes |
| Test type / Reverse or other mutation | Pos.  | Neg.  | Equiv.* | Pos.  | Neg.  | Equiv.* | “+” finding by Demerec et al. 1951. |       |
| Reverse or other mutation | 0/4 | 3/4 | 1/4 | 1b/7 | 5/7 | 1b/7 |       |
| DNA or hemoglobin adducts | 0/0 | 0/0 | 0/0 | 0/1 | 0/1 | 1b/1 |       |
| Cyto genetic damage | 0/2 | 2/2 | 0/2 | 0/3 | 2b/3 | 1b/3 |       |

*Indicates equivocal or weakly positive; includes results reported as equivocal, as well as results reported as positive if additional data make study interpretation equivocal as indicated in corresponding Notes.
A two-year study (Chun et al. 1992) was reported by Bird et al. (1997) in male and female F344 rats (50 animals per sex per group) exposed for 24 months to concentrations of 0, 400, 3,000, or 8,000 MTBE in air (details in Supplementary Table B-1). Parameters analyzed included hematology, BW, blood corticosterone levels, and urine at six months. Necropsy was performed, and brain, liver, kidneys, lungs, spleen, adrenals, and testes were weighed. All observed lesions were subjected to histopathological analysis. Histopathological analysis was also performed on 44 organs of the high-dose and control-group animals. Decreased BW gains were observed in the high-dose animals and chronic progressive nephropathy (CPN) was observed in all treated animals with the greatest severity at the highest two doses. In males, mean survival time and rate were decreased at the highest two doses, and at the low dose, survival was 27% compared with 17% for controls. Exposure-related decreases in male rat survival were attributed primarily to CPN; survival was not as severely affected in females. Increases in absolute and relative liver and kidney weights were observed in both sexes at 3,000 and 8,000 ppm. In the two highest dosed female groups, CPN was observed, albeit to a lesser extent than in males. Symptoms of CPN observed in males at all treatment levels included glomerulosclerosis, tubular proteinosis, and interstitial nephritis and fibrosis. Incidences of parathyroid adenoma and renal tubule tumors were increased in males in the two highest exposure groups, with renal adenoma and/or carcinoma significantly elevated in the 3,000-ppm exposure group. At the highest two doses in males, testicular Leydig interstitial cell adenomas were statistically significantly increased. Regarding these tumors, Chun et al. (1992) suggested that the observed “increase in the frequency of interstitial cell adenomas of testes may be a reflection of altered pituitary function, since a larger average size of the pituitary and adenomas was observed in the control and low concentration groups.” Additionally, it is possible that because of the high incidence of CPN in the two highest dose groups, and the corresponding decreases in mean survival time, the occurrence of Leydig cell interstitial cell adenomas in the study by Chun et al. appears artifactualy increased in the highest two dose groups. Furthermore, as Bird et al. (1997) concluded, in the presence of such high background occurrence in the aging rat, it is problematic to assign toxicological significance to the Leydig cell tumor (LCT) findings concerning the increased testicular tumors observed in this study:

This tumor is typically the most frequently observed spontaneous tumor in aged male Fischer 344 rats. The incidence observed in the MTBE-exposed groups were within the range (64–98%) previously reported for aged male Fischer 344 rats. The frequency of these testicular adenomas in control animals (64%) from this study was lower than that normally reported, and below the laboratory’s previous control values of 86% and 91%. Thus, the increased incidences of testicular tumors in the 3000 and 8000 ppm groups reflect the lower incidence in the control group and may not be treatment related. If the apparent dose-related incidence is a real finding, it may not necessarily be due to a direct action of MTBE but may be indirect, e.g. hormonal mediated.

Belpoggi et al. (1995), of the Ramazzini Institute in Italy, evaluated the chronic toxicity of MTBE in a lifetime assay in Sprague-Dawley rats, with oral administration of MTBE by gavage in olive oil for 4 days/week for 104 weeks of the lifetime study (60 animals/sex/group). Following cessation of dosing at 104 weeks, animals were allowed to live out the rest of their natural lives and were necropsied at the time of spontaneous death. Animals administered MTBE did not display adverse clinical signs, and BWs of treated groups were not significantly affected compared with control animals. Water and food consumption was comparable between controls and treated animals. Survival was adversely affected with dose—response and was evident at week 88 of the study. Survival was more adversely affected in males compared with females. There were no observations at necropsy that were not of a neoplastic nature. There were also no microscopic alterations that were not tumor related. The only lesions reported as statistically significantly elevated were testicular interstitial cell adenomas, which were significantly increased in frequency in males at the highest dose, and combined hemolymphoreticular tumors in females at both doses. On reevaluation by Belpoggi et al. (1998), however, only the testicular adenomas were reported to be statistically significant. Furthermore, with regard to the hemolymphoreticular tumors reported in studies conducted at the Ramazzini Institute, such as this study, the U.S. EPA (2012a) has determined that it will not consider data on soft tumors from the Institute in its carcinogenicity assessments due to conflicting assessments of data between pathologists at the Ramazzini Institute and an independent Pathology Working Group (PWG) of the NTP. Specifically, on reviewing data from the Ramazzini Institute, the NTP PWG did not reach the same conclusions as the Institute pathologists. Institute pathologists had reported greater incidence of soft tumors than reported by NTP PWG pathologists reviewing the same data.

In the most recent study of MTBE tumorigenicity, Dodd et al. (2013) administered MTBE to male and female Wistar rats at concentrations of 0, 0.5, 3, or (for males) 7.5, or (for females) 15 mg/mL in drinking water for 2 years. There were no significant effects of treatment on BW, but there was a significant reduction in water intake in all treated groups and sporadic differences in food consumption between treated groups and controls. There were no differences in survival between treated or control groups. There were no differences between organ weights of treated animals and controls, except for kidney weight, which increased significantly in all treatment groups of males and females. Related to this, CPN was observed in both males and females, albeit more severely in males and increasing in severity with dose. CPN reached statistical significance in males and females at the highest respective dose. The only finding of tumorigenicity in the study by Dodd et al. (2013) was an increased incidence of the rare tumor astrocytoma in males, which was reported as being marginally but (using a one-tailed statistical test for trend) significantly elevated at the highest dose compared with control rats in this particular study. The authors concluded that the occurrence of this lesion in the study was observed within the range of historical controls.

Relatively high doses of MTBE were shown to induce neurotoxicity and specifically astrocyte apoptosis in rats exposed for 60 days to doses from 0.5 to 500 mg/kg/day in vivo (Cherkasov et al. 2010), but this study did not include any quantitative dose–response analysis. Concentrations of ~1 mM.
of the MTBE metabolite formaldehyde can increase lactate and formate concentrations and alter glucose metabolism in rat astrocytes in vitro (Tulpule and Dingen 2012). However, MTBE concentrations as low as 0.34 mM significantly suppressed vascular endothelial cell tube formation and thus MTBE is now being considered as an anti-angiogenic treatment for solid tumors with minimal toxicity (Kozlosky et al. 2013).

For the observed incidence of astrocytomias in male Wistar rats (1/50, 1/50, 1/50, and 4/50, in the rat groups of increasing exposure, respectively), Dodd et al. (2013) reported that none of the exposed rat groups exhibited a significant positive response using a one-tailed Fisher’s exact test, and that a chi-square test assessing survival differences in the four groups of male rats indicated that there were no significant survival differences ($p = 0.59$). However, Dodd et al. (2013) reported that two trend tests yielded results considered significant: a one-tailed p value of 0.032 was obtained using a Cochran–Armitage test for linear trend (citing Cochran [1954] for the test performed), and a one-tailed p value of 0.037 was obtained using a version of this trend test modified by the “poly-3” procedure to adjust for survival differences. In contrast, two-tailed Fisher’s exact tests (Breslow and Day 1980) performed on the same data (using Mathematica 8.0® software, Wolfram Research 2012) for the present study indicated that none of the exposed rat groups exhibited a significant positive response ($p \geq 0.36$). The combined incidence data exhibit proportions of rats with astrocytomias that are homogeneous, in that they do not differ significantly by dose group ($p = 0.37$, by extended Fisher’s exact test [Baglivo et al. 1988]). Applying the numerically “exact” version of a two-tailed Cochran–Armitage trend test (Cochran 1954, Armitage 1955, Agresti 2002) implemented using Mathematica 8.0 software (Wolfram Research 2012), together with a standard criterion of $p \leq 0.05$ for assessing statistical significance, the reported astrocytoma data exhibit evidence of a positive trend with dose that is not statistically significant ($p = [141,721,628]/[1,631,354,439] = -0.087$). Using supplemental information from this study that 0, 1, 1, and 3 male rats died in each of the exposure groups before the first astrocytoma appeared in each of those groups, respectively (Dr. Darol Dodd, personal commun., 13 July 2012), the corresponding tumor incidences adjusted for intercurrent mortality are 1/50, 1/50, 1/49, and 4/47, respectively. These combined survival-adjusted tumor incidence data exhibit proportions of rats with astrocytomias that do not differ significantly by dose group ($p = 0.43$, by extended Fisher’s exact test), and a two-tailed exact Cochran–Armitage trend test again indicates that the astrocytoma data show no significant evidence of a dose-related trend ($p = 10,791,215,631/109,321,191,785 = -0.099$).

All rats in the Dodd et al. (2013) study experienced a very small inhalation exposure to MTBE due to volatilization from spilled water and from MTBE exhaled by exposed groups in the single room used for all control and exposed groups. The inhalation of MTBE by control rats could theoretically have increased tumor risk above spontaneous rates in the control animals (Burns and Melnick 2012). However, according to Dodd et al. (2013), “MTBE air concentrations in the animal rooms averaged less than 0.13 ppm with a single high reading of 0.24 ppm. Control cage MTBE air concentrations averaged less than 0.11 ppm.” Using the PBPK-based relationship shown in Figure 2 (Section 2.2.1), these background MTBE concentrations in air correspond to retained MTBE doses of approximately 0.01 mg/kg/day, that is, doses approximately equal to 0.1% of the MTBE dose retained by rats in the lowest exposure group. The background MTBE concentration in air in this study was thus negligible in the control group as well as in the exposed groups. Consequently, there is no plausible basis to expect that the background air concentration of MTBE experienced by the control group of male rats in this study could have contributed to any detectable dose-related elevation in the rate of astrocytoma incidence in that group.

Dodd et al. (2013) also reported multiple cases of pododermatitis in all exposure groups including controls beginning at week 56 of their study, occurring principally in male rats and including nine cases so severe that affected rats were euthanized. This condition (“bumblefoot”) involves footpad swelling and infection that is commonly observed in caged, overfed, or otherwise poorly husbanded rats, hamsters, rabbits, guinea pigs, and birds (Blair 2013). Other actions taken to reduce the incidence and severity of pododermatitis included “increased frequency of bedding changes, change of bedding type from Alpha-Dri to aspen shavings, and providing enrichment in the form of cardboard tubing” (Dodd et al. 2013), as well as topical applications of both an aseptic solution (chlorhexidine) and an antibiotic (triple antibiotic ointment) to the infected area (Burns and Melnick 2012). Possible related confounding effects on astrocytoma incidence in this study are unknown.

It is noteworthy that the most sensitive adverse endpoints identified among all those associated with chronic exposure to MTBE were observed in the bioassay study reported by Dodd et al. (2013). This study showed a significant increase in weight gain relative to BW (but, except in the group exposed to the highest MTBE concentration, not absolute weight gain) observed in both kidneys of male rats. At all non-control concentrations tested, this study also showed a significant increase in the absolute (but, except in the group exposed to the highest MTBE concentration, not relative) weight of the left kidney, but (except in the group exposed to the highest MTBE concentration) not in the right kidney, of female Wistar rats exposed to MTBE in drinking water for two years (Dodd et al. 2013).

Burleigh-Flayer et al. (1992) studied and Bird et al. (1997) reported on the tumorigenicity of MTBE in an 18-month study in male and female CD-1 mice (50/sex/dose). The study protocol was the same as that of the study conducted by Bird et al. (1997) in rats, except for the shorter, 18-month duration of study. Mortality was increased and mean survival time decreased in males treated at the highest dose, likely a result of slightly increased incidence of obstructive uropathy. Neurological clinical signs were observed in both sexes as a result of treatment. Body weight gain was decreased in males and females at the highest dose. Hematological parameters were not altered as a result of treatment; however, increased corticosterone was observed at the highest dose in males and females at 79 weeks. There was a slight decrease in urinary pH in high-dose males and females and a slight increase in gamma-globulin fraction in males at the same dose. Increases in absolute liver weight and in liver weight relative to BW were observed in a concentration-dependent manner in males and females. Increases in absolute kidney weight were also
observed in males at all treatment levels and in females at the highest dose. Increases in kidney weight for male mice, however, were not concentration dependent. Absolute brain and spleen weights were decreased in males and females at the highest dose. The only neoplastic or non-neoplastic lesions microscopically observed in the study were of the liver. Female mice were noted to have a significantly increased incidence of hepatocellular adenoma (compared with controls) only at 8,000 ppm, the highest dose, which was considered by the study authors to have exceeded the maximum tolerated dose (MTD) of MTBE in this study. Since the dose at which the increased incidence of hepatocellular adenomas occurred was above the MTD, the significance of this lesion to MTBE risk assessment was questioned. Although not reported by the study authors as significant, the study data (adjusted for rats dying before the first tumor occurrence, as reported in Table 9 [at p. 58] of CalEPA OEHHA 1999) show a statistically significant increase in male liver adenocarcinomas at the highest dose compared with controls \( (p = 0.036) \), here recalculated by two-tailed Fisher’s exact test using the CalEPA OEHHA adjusted incidence data), and a significant positive trend with dose \( (p = 0.018) \), by two-tailed exact Cochran–Armitage test for trend, here similarly recalculated but also using PBPK-based estimated doses listed in Table 3 in Section 6.2).

One additional study investigating chronic exposures involving MTBE was conducted by Benson et al. (2011). In this study, F344 rats were administered gasoline vapor condensate (GVC) with or without MTBE by inhalation. Male renal tumors and mononuclear cell leukemia were significantly elevated in the MTBE-exposed rats, but not significantly more so than in male rats exposed only to GVC. Male rats had higher incidence of testicular interstitial (Leydig) cell adenoma at the high dose in the GVC + MTBE group, but not in the GVC group. However, this was very likely an artifact of the unusually low incidence in the GVC + MTBE control group, 43/50, while the incidence in the GVC control group was 48/50 and more similar to all treated groups. Additionally, exposure levels were unrelated to the response pattern observed for this tumor type. The only tumor incidence that differed significantly between rats treated with GVC + MTBE and rats treated with GVC alone was an increased incidence of mammary gland fibroadenoma, which was significantly elevated above gasoline-only treated animals at the low and high dose. It is difficult to speculate on the significance of this finding, however, because the effect was not significant at the intermediate dose, the incidence in the corresponding control animals was low (8%) relative to the historical control range for this tumor (27–40%), and it is unclear how much this effect may be attributable to interaction with the much greater concentration of GVC used in this study. Finally, the results of this study are not corroborated by other studies regarding a mammary fibroadenoma response with chronic MTBE exposure.

In summary, chronic oral MTBE exposure increased renal tubule cell adenoma occurrence in male F344 rats, albeit by mechanism involving exposure-enhanced CPN, protein accumulation in kidney tubule, and early associated hyperplasia that are consistent with a recognized male-rat-specific MOA for this tumor type (see Section 5.2.1). The finding of lymphoma or leukemia by the Ramazzini Institute is questionable according to NTP PWG conclusions regarding inappropriate assignment of these tumors, and the mammary fibroadenoma finding is not corroborated by other studies and was derived from the use of a questionably relevant test article (MTBE + GVC). The finding of elevated astrocytoma was not statistically significant by two-tailed tests, and was also not corroborated by other studies. The observed increase in parathyroid adenomas was not statistically significant and considered not clearly translatable to humans. Finally, the finding of liver tumors in female mice has been postulated to be related to estrogenic or tumor-promoting effects of MTBE (Moser et al. 1996a, 1998); however, MTBE did not display tumor-promoting activity (Moser et al. 1996b; 1997), and no evidence exists to corroborate the theory of estrogenic alterations affecting the liver following MTBE treatment (EU 2002). The EU further concluded that the relevance of these tumors to man is questionable and noted that very high doses were needed to achieve this effect.

The only tumor types that unequivocally were significantly elevated by chronic MTBE exposure are therefore (1) testicular interstitial cell (Leydig cell) adenomas in Sprague–Dawley rats reported by Belpoggi et al. (1995, 1998), and in F344 rats by Chun et al. (1992) and Bird et al. (1997); (2) liver tumors in both sexes of CD-1 mice reported by Bird et al. (1997) and reanalyzed by CalEPA OEHHA (1999) and in the present study (see Section 4.1); and (3) renal tubule tumors in male rats reported by Bird et al. (1997) and perhaps also those reported by Benson et al. (2011). Possible MOAs for all MTBE-induced tumor types identified in this section are discussed in Section 5. In particular, the human toxicological significance of the elevated Leydig cell adenomas in rats has been questioned, while that of the elevated renal tubule tumors in male rats is dubious. MTBE-induced exacerbation of CPN, protein accumulation in proximal kidney tubule cells, and associated increased epithelial cell turnover are all likely to have contributed to elevating renal tubule cell tumors specific to male rats (see Section 5.2.1). The EU (2002) risk assessment report concluded that no definitive link can be made between LCT formation and chronic MTBE exposure. Several studies have sought to link disturbances in hormonal homeostasis with the occurrence of the LCTs following MTBE exposure, but no clear link has been established (see Section 5.2.4). Quite high background incidence of and scope of hormonally mediated effects on testicular Leydig cell tumors in aging male rats implies uncertainty concerning the human toxicological relevance of rat Leydig cell tumor findings, particularly for a cancer potency assessment that (by definition) assumes increased tumor risks are linearly proportional to tissue concentrations at very low doses (see Section 5.2.4).

4.2 Formaldehyde

There is sufficient evidence according to IARC (2012) to state that formaldehyde is carcinogenic via inhalation and IARC classifies formaldehyde as a Human Carcinogen. Several studies demonstrate carcinogenic effects of formaldehyde at sites of initial contact with the administered dose. These “point-of-contact” effects appear to result from direct contact with relatively high local concentrations of formaldehyde, and it does not necessarily follow that formaldehyde formed endogenously via intracellular metabolism of MTBE would have the same
formaldehyde induces nasopharyngeal tumors and leukemia more consistent with a nonlinear MOA for formaldehyde—The noted epidemiological associations were stronger for peak exposure and cumulative exposure, where all eight exposed cases were in the highest category of peak exposure (RR = 1.83) and “weaker exposure–response relationships were observed between nasopharyngeal cancer and average or cumulative exposure, and duration of exposure.” Concerning formaldehyde risk for leukemia, IARC (2012) cited positive associations reported in several cohort studies (e.g., Hauptmann et al. 2003, 2004, 2009) and four meta-analyses (e.g., Zhang et al., 2009), and also noted that … some evidence for an excess of deaths from leukaemia has been reported in the recent updates of two of the three major cohort studies of industrial workers … As in the previous analysis of leukaemia, the association in the most recent update was stronger for myeloid leukaemia and peak exposure than for lymphatic leukaemia and for other metrics of exposure … Additional analyses with time since first exposure and time since first high peak-exposure indicated that risks were highest during the first twenty-five years. Patterns were similar, but attenuated, for average intensity of exposure; no association was observed with cumulative exposure.

The noted epidemiological associations were stronger for peak exposures than for average or cumulative exposures, and are more consistent with a nonlinear MOA for formaldehyde-induced tumors. Epidemiological studies indicating that formaldehyde induces nasopharyngeal tumors and leukemia in humans are based on relatively few cases, involve marginally elevated relative risks of -2 or less, are ambiguous in view of potential confounding factors, are contradicted by large studies indicating no elevated risk, and are still debated (Pyatt et al. 2008, Golden 2011). However, the 2012 IARC Working Group “was not in full agreement on the evaluation of formaldehyde causing leukaemias in humans,” concluding that the “possibility of a mutagenic effect of formaldehyde on circulating lymphocytes or local lymphatic tissue cannot be excluded,” based on experimental results of Zhang et al. (2010) indicating that formaldehyde is potently cytotoxic to circulating hematological precursor cells and that occupational exposures may be associated with aneuploidy characteristic of myeloid leukemia and myelodysplastic syndromes—results that the study authors and the Working Group felt needed to be replicated (2012a, p. 30).

Potential associations between formaldehyde and hematolymphopoietic and solid cancers had been investigated in studies by Hauptmann et al. (2003, 2004) of 25,619 U.S. formaldehyde workers who had been employed in 10 different plants through 1994. Elevation of nasopharyngeal cancers was most significantly associated with the highest peak exposure (p for trend < 0.001), and formaldehyde exposure was not significantly/consistently associated with lung, pancreas, brain, or prostate tumors (Hauptmann et al. 2004). Evidence Hauptmann et al. (2003) showed for elevated hematolymphopoietic cancers was deemed limited by IARC, and showed the strongest association with peak exposures, but not when cumulative exposure was considered. Further, upon updating the cohort to include deaths and 10 years of new data, the association with leukemia weakened and cumulative exposure to formaldehyde showed no relationship to hematolymphopoietic cancers. In addition, a UK cohort that had the highest exposure showed a deficit in this cancer type (Coggon et al., 2003). Conversely, in a study that did not use actual formaldehyde measurements, Hauptmann et al. (2009) reported that higher leukemia incidence was associated with the number of work years for persons in the embalming industry, but did not find such an association for either brain or hematolymphopoietic cancers.

In the NCI cohort reported by Hauptmann et al. (2003), nasopharyngeal cancers (NPC) were elevated specifically in workers located at one of the 10 plants where six of the 10 total NPC cases were found—as independently confirmed by Marsh et al. (2007) who pointed out that in these workers with NPC, there was a work history of silversmithing, brass plating, and metal work, involving exposure to vaporized acids, which is independently associated with NPC. A review by Swenberg et al. (2013) cited several additional studies showing little support to conclude that formaldehyde causes NPC noted that NPC was also not elevated in two studies addressing the other largest two cohorts of formaldehyde workers (Coggon et al. 2003, Pinkerton et al. 2004), and concluded that although the overall data for human exposure are limited, there is biological plausibility to the argument that formaldehyde exposure results in NPC.

Heck and Casanova (2004) reviewed evidence indicating that inhalation of formaldehyde failed to induce leukemia in any of seven long-term inhalation bioassays in rats, mice, or hamsters. A review by Checkoway et al. (2012) concluded that epidemiological data linking exogenous formaldehyde exposure to hematolymphopoietic cancers is inconsistent and that available mechanistic data do not support such a link. As noted above, IARC (2012) did not reach a consensus in its evaluation of the hypothesis that formaldehyde causes leukemias in humans. Experimental results reported Zhang et al. (2010) were subsequently challenged by Gentry et al. (2013), but more recent reports of systemic formaldehyde effects (Ji et al. 2014, Yu et al. 2014, see Section 3.2), as well as evidence of methanol tumorigenicity (Section 4.4), suggest at least some plausible basis for potential tumorigenic effects of excess formaldehyde that is either systemically delivered or metabolically generated (e.g., after MTBE exposure). Primary mechanistic challenges to this hypothesis focus on contrary observations that after inhaled formaldehyde is systemically distributed, no elevations in DNA adducts, in unambiguous genotoxic damage, or even in blood levels of formaldehyde have been detected in vivo (Speit et al. 2009, 2010, 2011b; Moeller et al. 2011, Lu et al. 2010, Kleinnijenhuis et al. 2013, Swenberg et al. 2013). Gentry et al. (2013) points out the conjecture that myeloid leukemia risk may be increased by formaldehyde-induced aneuploidy requires that such effects must occur unambiguously in
circulating proliferating hematopoietic stem cells in vivo, and that such affected cells must then return to the bone marrow to generate that tumor type, and they review experimental evidence inconsistent with these assumptions.

As discussed in relation to genotoxic effects of formaldehyde (Section 3.2), mitochondrial FDH (ADH3) and GSH effectively and efficiently detoxify formaldehyde formed by intracellular metabolism, including normal metabolism in the absence of MTBE exposure and oxidative MTBE metabolism during and after MTBE exposure. Furthermore, the P450-dependent process by which formaldehyde is formed from MTBE can become saturated, limiting the amount of formaldehyde that can be formed, in contrast to GSH-dependent formation of formaldehyde from other small molecules such as methylene chloride (Andersen et al. 1987, Casanova and Heck 1997). These observations are consistent with profound nonlinearity observed by Monticello et al. (1996) in the dose–response relationship for formaldehyde-induced nasal tumors in rats (Figure 11), the pattern of which correlates with statistically significant increases in cell proliferation index they measured in the 10- and 15-ppm formaldehyde groups relative to control rats (observed most predominantly in the anterior lateral meatus and the medial maxilloturbinate areas of the nasal mucosa), which they attributed to regenerative hyperplasia.

Formaldehyde-induced regenerative cell proliferation in rat nasal epithelium has long been recognized to occur (e.g., Chen et al. 1983b). Meng et al. (2010) examined mutations and cell proliferation in nasal epithelial tissue of male F344 rats exposed to 0, 0.7, 2, 6, 10, or 15 ppm of formaldehyde for 13 weeks (6 h/day, 5 days/week). No dose-related increase in either p53 or K-Ras mutations was detected in this study, but the percentage of bromodeoxyuridine (BrdU) labeled (proliferating) cells in the interior lateral meatus was elevated but the percentage of bromodeoxyuridine (BrdU) labeled (proliferating) cells in the interior lateral meatus was elevated in all exposure groups relative to control rats (observed most predominantly in the anterior lateral meatus and the medial maxilloturbinate areas of the nasal mucosa), which they attributed to regenerative hyperplasia.

Figure 11. Data of Monticello et al. (1996) on the probability of nasal tumor occurrence (open points) in rats exposed for 6 h to different air concentrations of formaldehyde. Error bars = ± 1 SE assuming binomial sampling error. Nonlinear models fit to the data are cubic (solid curve), threshold (dashed curve), and Gaussian (dotted curve) functions of concentration.

Studies of the carcinogenicity of formaldehyde, or formalin (a commercial aqueous solution of formaldehyde), administered in drinking water present inconclusive evidence concerning whether formaldehyde is carcinogenic by this route (U.S. EPA 2010). In the study by Soffritti et al. (1989), increased incidence of tumors of the forestomach, gastrointestinal (GI) tract, and leukemia were observed in male and female Sprague–Dawley rats exposed for 104 weeks to 0 or between 10 and 2,500 mg/L of formaldehyde in drinking water. EPA noted that these study data were limited and the pathology lacked independent review (U.S. EPA 2010). Regarding the reported increased leukemia incidence in this study, EPA currently no longer considers data on lymphomas and leukemias from studies conducted at the Ramazzini Institute in its IRIS risk assessments, because an independent NTP review of histopathology data from that Institute identified fewer lymphomas and leukemias than reported by the scientists at the Institute (U.S. EPA 2012a). In contrast to findings reported by Soffritti et al. (1989), a study of paraformaldehyde administered to male and female Wistar rats in drinking water, corresponding to doses of up to 82 and 109 mg/kg/day, respectively, showed no evidence of carcinogenicity (Til et al. 1989).

Oral studies by Takahashi et al. (1986) (8 weeks of MNNG initiation followed by 32 weeks of 0.5% formalin in drinking water consumed by male outbred Wistar rats) and by Soffritti et al. (1989) (described above) both reported neoplastic lesions of the GI tract following chronic administration of formaldehyde through drinking water, and these are also summarized in Supplementary Table B-2. The total incidence of benign plus malignant GI neoplasms listed in Table 20 of Soffritti et al. (1989) indicate statistically significant dose-related trends for these tumors in male rats (p = 0.0049) and in female rats (p = 0.046) by exact Cochran–Armitage trend tests (calculated as indicated in Section 4.1). As with the lesions observed...
resulting from tissue contact in the nasal epithelium, a drinking water exposure route is not relevant to formaldehyde exposure resulting from metabolism of MTBE to formaldehyde, as such metabolism would occur intracellularly at mitochondrial sites rich in antioxidants and FDH (ADH3), and not as an external concentration gradient accessible to other, less protected cellular sites.

Concerning formaldehyde cancer risk to humans, the German Federal Institute for Risk Assessment (BfR 2006) and Golden (2011) concluded that protection against cytotoxic respiratory effects protects against possible cancer risk posed by respiratory formaldehyde exposure. According to the German Federal Institute for Risk Assessment (BfR 2006):

… there is evidence that a relevant increment of DPX formation is linked to intracellular glutathione depletion at higher exposure concentrations, which leads to a non-linear increase of intracellular formaldehyde, and to sustained cell proliferation due to cytotoxic effects. The proposed mode of action is based on observations of consistent, parallel dose-response relationships for all three (sustained cell proliferation, generation of DPX and tumors) and on the concordance of the incidence of these effects across various regions of the nasal passages of the rat. While exposure to formaldehyde leads to both genotoxic effects and irritation/cytotoxicity, neoplastic transformation is observable only if certain concentrations in the target cells are reached or exceeded, leading to a non-linear dose-response relationship which is mainly determined by regenerative hyperplasia. The concentration-response relationships for DPX formation, cytotoxic effects, proliferative response and tumors are highly non-linear, with a significant increase of the slopes at concentrations of around 4 ppm, a concentration at which glutathione-mediated metabolism is saturated (Casanova and Heck 1987). According to data from Swenberg et al. (1983) and Casanova et al. (1994), increased epithelial cellular proliferation, histological changes and DPX formation are more closely related to exposure concentration than to total cumulative exposure. This finding is in line with the analysis of exposure matrices in the epidemiological studies where the observed tumors were related to peak exposure (Hauptmann et al., 2004).

… As pointed out irritation/cytotoxicity is the most important step in the chain of events. Hence, the NOAEL for this effect has been used as point of departure for the derivation of the “safe” level. … from the available data a NOAEL of about 0.1 ppm may be derived. … The derivation is in agreement with the evaluation of WHO (2002) that only a very small proportion of the population experience symptoms of irritation following exposure to 0.1 ppm formaldehyde. … As the point of departure for deriving the “safe” level is based on human data, no interspecies safety factor is needed. The level of 0.1 ppm is more than 10 times lower than a threshold for cytotoxic damage to the nasal mucosa. Hence, we conclude that a safety factor accounting for intraspecies variability is not necessary. Given the described mechanism of action for tumor formation, the formaldehyde concentration of 0.1 ppm represents an exposure level at which there is practically no risk of upper respiratory tract cancer in humans.

In its review of the EPA draft IRIS assessment of formaldehyde, the National Research Council (NRC) concluded (NRC 2011, p. 4–5):

Humans and other animals produce formaldehyde through various biologic pathways as part of normal metabolism. Thus, formaldehyde is normally present at low concentrations in all tissues, cells, and bodily fluids. … Formaldehyde is a highly water-soluble, reactive chemical that has a short biologic half-life. Despite species differences in uptake due to differences in breathing patterns and nasal structures, formaldehyde is absorbed primarily at the site of first contact where it undergoes extensive local metabolism and reactions with macromolecules. Thus, the net result is that inhaled formaldehyde remains predominantly in the respiratory epithelium that lines the airways. … the weight of evidence suggests that formaldehyde is unlikely to appear in the blood as an intact molecule except perhaps at concentrations high enough to transiently overwhelm the metabolic capability of the tissue at the site of exposure. Thus, direct evidence of systemic delivery of formaldehyde is generally lacking. … data are insufficient to conclude definitively that formaldehyde is causing cytogenetic effects at distant sites. … the data strongly suggest that formaldehyde is not available systemically in any reactive form.

… EPA based its approach to its cancer assessment primarily on the conclusion that formaldehyde is a genotoxic chemical that causes mutations (a mutagenic mode of action). However, for nasal tumors attributed to formaldehyde exposure, animal data also support a mode of action characterized by regenerative cellular proliferation that results from cytotoxicity. Because multiple modes of action may be operational, the committee recommends that EPA provide additional calculations that factor in regenerative cellular proliferation as a mode of action, compare the results with those presented in the draft assessment, and assess the strengths and weaknesses of each approach.

4.3 TBA

Studies of the chronic toxicity or carcinogenicity of TBA were limited to one study conducted by the NTP (1995) that was published as Cirvello et al. (1995). In this study, male and female F344 rats and B6C3F1 mice were administered TBA via drinking water for 103 weeks. The results of the study in mice and rats are summarized below and in Supplementary Table B-3 (in Supplementary Appendix B to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367).

Survival was significantly decreased in male and female rats at the highest dose levels. The final mean BW of high-dose males and females was significantly lower than controls. The BWs of other treated groups were similar to controls. Hyperactivity in high-dose female rats was the clinical sign reported. A few minor or sporadic changes were observed in hematology and urinalysis; however, these were not considered to be treatment related. Renal tubule hyperplasia and mineralization was observed with dose dependence in treated males although not all observations reached significance. Nephropathy was observed with significantly increased severity in all dosed females and in males at the highest dose.

Significant neoplastic observations were limited to increased incidence of renal tubule cell adenomas in male rats, and to decreased incidence in female mammary gland tumors and in male pancreatic islet cell tumors. Significantly increased renal tumors were not observed in the treated female
rats. NTP (1995) concluded that based on the histopathology of the kidney, alpha2u-globulin accumulation was likely a factor in the development of renal tubule cell hyperplasia and tumorigenesis in male rats. Since both male and female rats also displayed a dose-related increase in CPN, NTP (1995) concluded that alpha2u-globulin accumulation could not account entirely for the observed TBA-induced exacerbation of CPN. Alpha2u-globulin accumulation as a MOA for chemically induced renal tubule adenomas in male rats is discussed further in Section 5.2.1.

Kidney toxicity and tumorigenesis observed in the rats were not observed in mice in the NTP (1995) study. Increased incidence of neoplasms in mice was confined to the thyroid. Combined follicular cell adenoma and carcinoma were marginally increased in male mice in the middle-dose group only; in female mice, the incidence rate of follicular cell adenomas of the thyroid was reported as being significantly increased in the highest-dose group (9/50) compared with that in controls (2/58) by a Fisher’s exact test with \( p = 0.023 \), which evidently reflects a one-tailed test, because the two-tailed \( p \) value for this test given these data is not significant \( (p = 0.053) \). However, the combined female data for this tumor exhibit a significant positive trend with dose \( (p = 0.010, \text{by two-tailed exact Cochran–Armitage trend test}) \). The incidence of hepatocellular adenoma in the high-dose group (12/59) was highly significantly reduced compared with that in controls (25/60) and all groups showed a significant negative dose–response trend, but this pattern was not evident either for hepatocellular carcinoma or combined hepatocellular adenoma or carcinoma (HAC); however, HAC was significantly reduced in the highest-dose group of male mice and showed a significant negative trend with dose. The incidence of combined adenoma or carcinoma of the Harderian gland was significantly decreased in all exposed males (6/30, 3/60, 10/60) compared with the control rate (1/60), and showed a significant negative trend.

4.4 Methanol

Three published chronic carcinogenicity studies of methanol have been conducted; one in mice and two in rats, as summarized in Supplementary Table B-4 (in Supplementary Appendix B to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Only one of these studies reported significantly increased tumor incidence.

The New Energy Development Organization (NEDO) in Japan conducted two chronic studies to determine the carcinogenicity of methanol (NEDO 1987, published as Katoh 1989). In these studies conducted using male and female F344 rats exposed for two years and B6C3F1 mice exposed for 18 months, no evidence of significant exposure-related tumorigenicity was reported. However, a U.S. EPA review of English-language translations of these studies concluded that the male rats in the highest methanol concentration (1000-ppm) group had a significantly elevated incidence of combined pulmonary adenoma/adenocarcinoma (PAC) (reporting \( p < 0.05 \) by Fisher’s exact test) as well as a significant dose-related trend, and female rats in this study showed a significant dose-related trend for adrenal medulla pheochromocytoma (PCC) incidence with the incidence rate in the highest exposure group significantly elevated above historical control rates for female F344 rats (though not for the female control rats in this study) (U.S. EPA 2009). The EPA review reported PAC incidence rates of 7/52 and 1/52 in the high-exposure and control male rats, respectively, which is not clear evidence of a significant incidence rate difference \( (p = 0.05986, \text{by the two-tailed Fisher’s exact test calculated in the usual manner, by summing over all hypergeometric likelihoods no greater than that associated with the two observed rates being compared}) \). The corresponding dose–response trend for PAC is only marginally significant when assessed by the exact two-tailed Cochran–Armitage test for trend \( (p = 0.045) \). Thus, while NEDO rat study data exhibit evidence of dose-related trends for lung nodules and for PAC in male rats, and for PCC in female rats, there were no significant pairwise differences in tumor rates in exposed versus the corresponding concurrent control rats. The EPA review concluded that the 18-month NEDO bioassay of methanol-exposed B6C3F1 mice showed no clear evidence of tumorigenicity, but that the rat bioassay results and other data supported a (to date, still draft) designation of methanol as a likely human carcinogen. The U.S. EPA (2012a) subsequently questioned the validity of certain formaldehyde carcinogenicity data it had relied on partly to arrive at this conclusion concerning methanol, as discussed below.

The only other published study reporting carcinogenic activity for methanol was conducted by Soffritti et al. (2002) at the Ramazzini Institute in Italy. Soffritti et al. observed higher incidence of “hemolymphoreticular tumors” in female rats but not in males. Additionally, ear duct carcinomas and head/neck sarcomas/fibrosarcomas were reported to be increased. The cancer “hemolymphoreticular” tumor consists of the sum total of all lymphomas and leukemias combined into one incidence value. Although this method of statistical analysis is commonly used by the Ramazzini Institute for soft tumor incidence determination, this is an unconventional method of calculating tumor statistics and is not generally considered appropriate or biologically meaningful. As a result, the observation of increased hemolymphoreticular tumors in female rats is of questionable significance. Furthermore, EPA has concluded for methanol, as it has also concluded for formaldehyde and MTBE, that soft tumors including lymphomas and leukemias reported by the Ramazzini Institute will not be used in risk assessments in support of the EPA IRIS program (U.S. EPA 2012a). This decision was based on evaluations by the NTP PWG who examined histopathological data generated at the Ramazzini Institute. Specifically, upon reviewing the same data indicating bias or inherent problems with soft tumor assignment by Ramazzini Institute scientists, the NTP PWG found fewer incidences of various tumors, including ear duct carcinomas and hematopoietic tumors, than did pathologists at the Ramazzini Institute (NTP 2011, U.S. EPA 2012a). The NTP PWG pathology review generally indicated fewer (in some cases, substantially fewer) tumors of various types than those reported in the Ramazzini Institute methanol study, including all those types reported as being significantly elevated in male or female rats; the revised NTP tumor counts do not indicate significant elevations of any tumor type (see Tables 5, 6a, and 6b of NTP 2011). The NTP reviewers also noted, “The findings suggest that the male and female rats in this lifetime drinking water study had a respiratory infection” (NTP 2011, Table 7), which calls into question the validity of
this study for the purpose of carcinogen classification—a conclusion supported by other toxicologists who have reviewed this methanol bioassay (Cruzan 2009, TERA 2010).

4.5 Formic acid

Chronic or carcinogenicity studies involving formic acid, which under physiological conditions appears as the conjugate formate ion, were not located; however, a 13-week inhalation study has been conducted by the NTP (1992). In this study, male and female F344 rats were administered inhaled doses of formic acid vapor by whole-body exposure. There were no deaths, clinical signs of toxicity, or adverse BW effects. Hematological changes were mild to minimal and no neoplastic lesions were observed. The only histopathological finding in the study was alteration of the respiratory and olfactory epithelium, which was generally limited to the highest-dose group. The NTP concluded that this finding was consistent with the irritant and acidic nature of formic acid and that there was no evidence of systemic toxicity.

4.6 2-Methyl-1,2-propanediol and Alpha-hydroxyisobutyric acid

Chronic or carcinogenicity studies of 2-methyl-1,2-propanediol or alpha-hydroxyisobutyric acid were not located.

4.7 Conclusions regarding MTBE tumorigenicity

Only three tumor types were unequivocally elevated after chronic exposure to MTBE in experimental rodent bioassays. These tumor types are (1) male rat renal (kidney) tubule cell tumors in F344 rats, (2) male rat testicular Leydig interstitial cell tumors in SD and F344 rats, and (3) male and female CD-1 mouse liver tumors. The MTBE metabolite TBA also induced kidney tumors in male F344 rats. The MTBE metabolite formaldehyde is, when administered exogenously, a recognized human carcinogen at sites of first contact of nasopharyngeal tissues with inhaled formaldehyde, and may cause leukemia via a mechanism possibly involving formaldehyde contact with nasal respiratory mucosal lymphatic tissue rather than systemically delivered formaldehyde (IARC 2012). However, these conclusions remain questionable in view of unresolved uncertainties and inconsistencies concerning the underlying epidemiological data and toxicological evidence that inhaled formaldehyde does not increase endogenous formaldehyde levels in systemic circulation nor does exogenous formaldehyde induce DNA adducts or crosslinks in tissue beyond the nasal epithelium (Lu et al. 2010, Golden 2011, NRC 2011, Speit et al. 2011b; Kleinnijehuis et al. 2013). Additional evidence that formaldehyde is not involved in observed MTBE-induced tumors in chronically exposed rodents is the absence of induced tumors—like those observed by in rats and humans chronically exposed to formaldehyde—in nasal tissue, where enzymes that metabolize MTBE to formaldehyde are present (Section 2.1.1). Uncertainty concerning possible tumorigenicity of formaldehyde generated as a metabolite is suggested by limited evidence for increased rates of lung tumors in male F344 rats and pheochromocytomas in female F344 rats that for two years inhaled air containing up to 1,000 ppm of methanol (Section 4.4), which like MTBE is metabolized to formaldehyde (Section 2.1.6). However, the parallel study using similarly exposed male and female B6C3F1 mice showed no clear evidence of tumorigenicity, including in the liver (Section 4.4). Previous agency classifications of MTBE carcinogenicity (summarized in Table 2) are consistent with these overall conclusions.

5. MOAs for tumors elevated by chronic exposure to MTBE and/or its metabolites

Estimation of MTBE cancer potency requires consideration of its carcinogenic MOA, which EPA has defined as a sequence of key events and processes (observable precursor steps, or associated biologically based markers), from interaction of an agent with a cell, proceeding through operational and anatomical changes, culminating in cancer formation (U.S. EPA 2005a, p. 1–10). This definition indicates that carcinogenic MOAs may include mutagenicity, mitogenesis, inhibition of cell death, cytotoxicity with reparative cell proliferation, and immune suppression. Careful consideration of carcinogenic MOA is required because the dose–response relationship for increased cancer risk that may be associated with an environmental chemical exposure typically is observed only at relatively high levels at which statistically convincing evidence of carcinogenicity can be obtained. For practical reasons that arise from limited statistical power when studying populations of relatively small size, such observations of significantly increased risk are rarely, if ever, made at levels of increased risk that are less than 1%—except, perhaps, in very large and well-designed clinical epidemiology studies involving pharmaceutical effects. Consequently, conclusions concerning the existence or magnitude of lower levels of increased risk inevitably depend on assumptions concerning the shape of the dose–response relationship, for those relationships that include an observed increase in risk that is statistically significantly elevated above that for an unexposed or “control” population. Information on how a chemical causes cancer (i.e., its MOA) contributes biological evidence to support statistically significant observations, and provides insight on the nature of the dose–response relationship at lower doses, below exposures associated with statistically significant increases in cancer risk.

For most forms of chemically induced toxicity, toxicologists have traditionally assumed that if the dose of any chemical is sufficiently low, that dose will pose no or only a negligible, or de minimis, risk of toxicity occurrence. This traditional approach corresponds to observations that “classical” forms of chemically induced toxicity, from cell killing to tissue toxicity to lethality, generally exhibit a nonlinear S-shaped (sigmoidal) dose–response relationship, consistent with the assumption that risk of any such toxicity can be reduced to zero or to de minimis levels by a sufficient reduction in dose (Paracelsus [Temkin 1941, Deichmann et al. 1986]; Aldridge 1986, U.S. EPA 2002, Hodgson 2004, Kimmel et al. 2006, Casarett et al. 2008). The traditional approach does not apply to toxic effects that accumulate over time (such as chemically induced mutations, or background mutations arising from synthesis errors), or effects that are amplified when a sufficient dose triggers a cascade response (e.g., when blood is induced to clot, or when the immune sys-
| Agency                        | Year | Comments                                                                                                                                                                                                 |
|------------------------------|------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NTP                          | 1995 | Some evidence of carcinogenic activity of TBA in male F344/N rat kidney (renal tubule adenoma & carcinoma combined), and in female B6C3F1, mouse thyroid (follicular cell adenoma or carcinoma combined); increased hyperplasia was observed at both tumor sites. |
| ATSDR                        | 1996 | “Weight of evidence suggests that effects of MTBE on the immunological or lympho-reticular system are not a concern for humans”; “Information regarding genotoxic effects of MTBE indicates that it has little if any genotoxic activity.” |
| National Science and Technology Council | 1997 | “Sufficient evidence that MTBE is an animal carcinogen”; “the weight of evidence supports regarding MTBE as having a carcinogenic hazard potential for humans” |
| U.S. EPA                     | 1997 | “Carcinogenicity data support a conclusion that MTBE poses a potential for carcinogenicity at high doses. The data do not support confident, quantitative estimation of risk at low exposures.” The weight of evidence indicates that MTBE is an animal carcinogen, and the chemical poses a carcinogenic potential to humans” |
| WHO IPCS                     | 1998 | “MTBE should be considered a rodent carcinogen” that “is not genotoxic and the carcinogenic potential is only evident at high levels of exposure that also induce other adverse effects … available data are inconclusive and prohibit their use for human carcinogenic risk assessment until outstanding complications in their interpretation have been addressed.” |
| NTP                          | 1998 | Not “reasonably anticipated to be a human carcinogen”                                                                                                                                                     |
| CalEPA OEHHA Prop. 65 Hazard Identification Document | 1998 | “There is evidence for the carcinogenicity of MTBE in animals” … “However, uncertainties remain about the nature and extent of risk at very low doses, and about the particular tumor sites that are most relevant to humans” |
| NTP                          | 1998 | NIEHS Report on Carcinogens (RoC) Review Group recommended that MTBE be listed in 9th RoC as reasonably anticipated to be a human carcinogen, based on evidence of benign and malignant tumor induction at multiple organ sites in long-term studies in two animal species (kidney and testicular tumors in male rats and liver tumors in mice, and combined leukemia and lymphomas in female rats and testicular tumors in male rats); NTP Executive Committee Interagency Working Group for the RoC defeated a recommendation to list MTBE in the 9th RoC as reasonably anticipated to be a human carcinogen, indicating that available rodent cancer data were not strong enough for such a listing because observed kidney tumors in rats and liver tumors in mice may have arisen by mechanisms not relevant to humans, and that there were no supporting human data. |
| CalEPA Public Health Goal     | 1999 | “Lack of evidence of cancer causation in humans is also a significant limitation”; “While there are varying degrees of uncertainty as to the relevance to human cancer causation … MTBE should be considered a possible human carcinogen” |
| International Agency for Research on Cancer (IARC), Vol. 73 | 1999 | Group 3: “inadequate evidence in humans for carcinogenicity”; “limited evidence” for carcinogenicity in animals                                                                                      |
| EU                           | 2002 | “There is insufficient evidence for MTBE to be classified as a carcinogen … there is at present no need for further information and/or testing and for risk reduction measures beyond those which are being applied already” |
| WHO                          | 2005 | any health-based guideline for MTBE would be significantly higher than the 15-μg/L concentration at which MTBE is detected by odor among test- and order-sensitive human subjects |
| U.S. EPA                     | 2009 | “a report from the National Toxicology Program (NTP) … reviewed results of some research studies completed by the Ramazzini Institute, a lab in Italy that conducts animal testing to evaluate the potential cancer-causing effects of chemicals. The report found differences of opinion between NTP scientists and the Ramazzini Institute (RI) in the diagnosis of certain cancers reported in a study on methanol.” … “EPA has decided not to rely on data from the RI on lymphomas and leukemias in IRIS assessments. This decision will impact IRIS assessments under development for methanol, MTBE and ETBE.” [bold font added] |
| U.S. EPA                     | 2012b| EPA responded to requests for guidance by reviewing and updating an advisory for MTBE in December 1997. This Drinking Water Advisory … provides guidance to communities that may become exposed to drinking water contaminated with MTBE. The advisory recommends control levels that prevent adverse taste and odor (i.e. 20 to 40 parts per billion). Managing water supplies to avoid the unpleasant taste and odor effects at levels in this range also provides protection against any potential adverse health effects with a very large margin of safety. |
| IARC                         | 2012 | Formaldehyde is carcinogenic to humans (Group 1). “There is sufficient evidence in humans for the carcinogenicity of formaldehyde. Formaldehyde causes cancer of the nasopharynx. There is sufficient evidence in humans for a causal association of formaldehyde with leukemia. There is limited evidence in humans for a causal association of formaldehyde with sinonasal cancer. There is sufficient evidence in experimental animals for the carcinogenicity of formaldehyde.” “Local effects in the nasal passages, genotoxicity, and cell-proliferation rate appear to be the major determinants of nasal carcinogenicity after exposure to formaldehyde.” |

*Summary refers to MTBE unless specified otherwise.*

define expectations concerning the existence or magnitude of increased cancer risk associated with very low levels of chemical exposure.

Subsection 5.1 briefly discusses the EPA’s default linear risk extrapolation for MOA for genotoxic carcinogens and some recent challenges to its scientific plausibility. Section 5.2 then addresses tumorigenic MOAs relevant to MTBE.
and its metabolites, and Section 5.3 presents conclusions specifically concerning tumorigenic MOAs for MTBE and its major metabolite, TBA. Experiments to help identify a MOA for MTBE-induced tumors are discussed in Section 5.4.

5.1 EPA’s default linear risk extrapolation for chemical carcinogens with a presumed genotoxic MOA

In 1976, EPA became the first government agency to adopt guidelines for scientific evaluation of environmental chemical carcinogen risks and, further, to state that gains in public health (i.e., reductions in risks) would be balanced against social and economic concerns in making regulatory decisions, to the extent permitted by enabling legislation (Anderson et al. 1983). Those and subsequent cancer risk assessment guidelines adopted an LNT approach to extrapolate increased cancer risks posed by exposures to environmental chemical carcinogens. The somatic mutation theory of cancer provided—and continues to provide—the scientific rationale for EPA’s default application of LNT risk extrapolation to calculate upper bounds on increased risk, while acknowledging that the true value of such risk is unknown and may be as low as zero (Anderson et al. 1983, U.S. EPA 1986). These guidelines assume that exposure to environmental genotoxic chemicals may (in some respects similar to mutagenic and carcinogenic ionizing radiation) cause irreversible, somatically inherited (e.g., DNA) damage that adds to background levels of such damage that explain (at least in part) background tumor incidence rates, in accordance with the somatic mutation theory of cancer (see Supplementary Appendix C to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Since there is “no solid scientific basis for any mathematical extrapolation model relating carcinogen exposure to cancer risks at the extremely low levels of concentration that must be dealt with in evaluating environmental hazards” (Anderson et al. 1983), LNT risk extrapolation for chemical carcinogens has been applied as a plausible, conservative assumption to enable quantitative risk assessment (NRC 1983). Later, NRC (1994) recommended that EPA address uncertainty and inter-individual variability in predicted cancer risks (particularly in applications that require risk trade-offs), and emphasized (at pp. 124–125) that many substances “can be carcinogenic by mechanisms that do not involve direct covalent interaction with DNA at all, but [could] involve … altered cellular dynamics … [that] could augment … background carcinogenic processes by simply increasing the pool of cells that are susceptible to further transformation … as a regenerative response to cellular injury among surviving cells or to the cell-killing that occurs after exposure to highly toxic substances.” EPA’s subsequent revised carcinogen risk assessment guidelines retained a default LNT approach to extrapolate low-dose cancer risk, particularly for plausibly genotoxic carcinogens with no other identified MOA (U.S. EPA 2005a). Besides a minor change in calculation procedures used to implement LNT risk extrapolation (see Supplementary Appendix D to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367), the new guidelines allowed an alternative nonlinear approach to be used if justified by “sufficient” MOA-related supporting data, noting that “‘sufficient’ support is a matter of scientific judgment in the context of the requirements of the decisionmaker or in the context of science policy guidance regarding a certain mode of action” (U.S. EPA 2005a, p. 2–42).

As the pivotal basis of EPA’s default LNT approach to cancer-risk extrapolation for carcinogenic chemicals (Supplementary Appendix C), somatic mutation tumorigenesis theory faces two key caveats. First, the somatic mutation theory does not imply that LNT risk extrapolation from any observed set of tumor-response data provides an estimate of increased risk that is always, or even typically, accurate at low doses, even as an approximation. Second, the somatic mutation theory of carcinogenesis is not the only current competing theory of carcinogenesis. There are other theories that have substantial scientific support, and the somatic mutation theory has some important weaknesses. These two key caveats are summarized in Sections 5.1.1 and 5.1.2, respectively. More in-depth discussion of these issues appears in studies cited in these sections, and in extensive additional references cited in many of those studies.

5.1.1 A genotoxic moa does not necessarily imply low-dose linearity of increased tumor risk

The somatic mutation theory does not necessarily imply that the true magnitude of low-dose risk must be proportional to dose with a slope meaningfully estimated from data observed at high levels of exposure, even for genotoxic carcinogens. While an approximately LNT dose–response for induced DNA damage by chemical carcinogens is both expected and observed for types of damage that are inefficiently or slowly repaired or are predictably misrepaired (Lutz 1986b; Kitchin and Brown 1994), endogenous DNA damage (which occurs as a by-product of oxidative metabolism unrelated to any xenobiotic chemical exposure) is typically subject to rapid and highly efficient repair, as shown to be the case for formaldehyde in vivo (see Sections 2.1.4, 3.2). Consequently, at lower chemical exposures, small relative changes in the level of DNA damage are indistinguishable from endogenous background levels of DNA damage and are expected to produce a threshold-like, nonlinear dose–response for increased DNA damage and consequent DNA mutations. Clear examples of such threshold-like, nonlinear patterns of response include DNA damage induced in livers of rats exposed to 1,2-dimethyl hydrazine, as measured by the alkaline elution “comet assay” method (Kitchin and Brown 1996); thymidine kinase mutations in L5178Y tk(+/−) mouse lymphoma cells exposed in vitro to ethyl methane sulfonate (Lutz and Lutz 2009); micronuclei induced in human TK6 cells exposed in vitro to each of six clastogenic chemicals (colchicine, vinblastine sulfate, ethyl methanesulfonate, methyl methanesulfonate, ethyl nitrosourea, and methyl nitrosourea) using a highly sensitive assay that, for only one of the chemicals (bleomycin), detected an approximately LNT dose–response (Bryce et al. 2010); and micronuclei induced in human TK6 cells exposed in vitro to naphthalene without any added GSH (the reduced form of the natural endogenous antioxidant, glutathione), where all naphthalene-induced micronuclei, and all associated cytotoxicity was abolished completely at all naphthalene
concentrations tested (up to 500 μM) when 5 mM (a physiologically relevant concentration) of GSH was added (Recio et al. 2012). Many other examples call into question a general assumption that genotoxic dose–response always has an LNT dose–response pattern (Bolt et al. 2004, O’Brien et al. 2006).

Increased risk associated with each critical mutation posited by the somatic mutation theory is assumed typically to be amplified considerably by net proliferation (i.e., clonal expansion) of premalignant (hyperplastic, intermediate-stage) cell populations that are involved in tumor progression (Armitage and Doll 1957). This feature predicts that chemical exposure may increase cancer risk either by increasing the rate of critical somatic mutations, or by increasing the net proliferation rate of premalignant cells, or both, where net proliferation of premalignant cells may be increased either by increasing their rate of birth (i.e., division, or mitosis), or by decreasing the rate of their death or terminal differentiation, or by both effects (Moolgavkar 1983, 1988, Bogen 1989). Net-proliferative cytotoxic effects can thus amplify any increased risk that might specifically be attributable to induced genotoxicity. Importantly, such amplification can occur by large magnitudes that can be difficult or impossible to estimate reliably with limited mechanistic and time-to-tumor data (Bogen 2008). If induced net proliferation of premalignant cells occurs only above a characteristic threshold level of chemical concentration, then exposure to this chemical may increase cancer risk with a threshold-like or substantially nonlinear dose–response relationship (see references cited above in relation to NRC 1994). Chemically induced genotoxicity correlates well with induced cytotoxicity, which is why assays for the latter endpoints are typically used to define concentrations used to detect the former endpoint. Chemical induction of adaptive or regenerative cell proliferation is typically a multifactorial, genotoxic, and/or cytotoxic cellular response that is not expected to have an LNT dose–response. More generally, although an external carcinogen that acts additively with any already ongoing process implies that it must increase risk with an LNT dose–response pattern, if a nonlinear or threshold-like effect precipitates or radically augments this process, the LNT argument breaks down (Crump et al. 1976).

5.1.2 The somatic mutation theory of tumorigenesis may be incorrect

Since it was proposed shortly after the initial discovery of basic DNA structure and function (Armitage and Doll 1954, 1957), the somatic mutation theory of cancer has proven to be consistent with observations that mutated oncogenes in somatic or germ cells can increase background rates of single tumor types, as in hereditary retinoblastoma, or of multiple tumor types as occurs in Bloom syndrome, Fanconi’s anemia, ataxia telangiectasia, Werner’s syndrome, Cockayne’s syndrome, xeroderma pigmentosum, and other inherited DNA-repair-defect diseases, and in the inherited, autosomal-recessive chromosome-fragility disorder Bloom syndrome, which is due to a DNA-helicase gene mutation (e.g., Cairns 1981, German et al. 1984, van Brabant et al. 2000, McKinnon and Caldecott 2007, Caldecott 2008). However, observations apparently inconsistent with this theory continue to be reported and thus to motivate alternative theories. For example, activated and presumed dominant oncogenes such as ras are also found in cancer-free animals, in benign human colon tissue with little potential to progress, in otherwise indistinguishable cancers and metastases lacking any ras mutation, and in human tumor cells that spontaneously lost mutated ras but not tumorigenicity (reviewed in Duesberg et al. 1998). While the somatic mutation theory presumes that accumulated mutations involving a relatively small set of critical oncogenes typically explains carcinogenesis, genomes of adult cancer cells characteristically contain usually between 1000 and 10,000 somatic mutations (Stratton 2011), large subsets of which evidently occur in single, critical (“chromothripsis”) events rather than gradually over time (Stephens et al. 2011). The significant discrepancy (p = 0.00044) between the unit slope between log (cancer potency) and log(mutagenic potency) that is expected based on multistage cancer theory, and the value (± 1 SE) observed (0.59 ± 0.09) for 68 mutagenic rodent carcinogens was noted as being inconsistent with somatic mutation tumorigenesis theory (Bogen 1995a). Similarly, a comparison of potencies for inducing DNA adducts versus cancer, based on data for six adduct-forming rodent carcinogens, indicated that no quantitative correlation exists between these two endpoints, and that cancer occurrence was not predicted by the mere occurrence of DNA adducts above or below the level of endogenous DNA damage (Paini et al. 2011). In contrast, a high correlation is observed between chromosome damage requiring at least two double-strand DNA breaks (deletions and/or translocations) and human cancers (Cairns 1981, Hanahan and Weinberg 2000); genomic instability that features such damage (together with increased mutation rates) continues to be viewed as a key enabling characteristic that triggers hallmark tumor capabilities (Hanahan and Weinberg 2011).

The aneuploidy theory—the oldest theory of cancer, first hypothesized by Theodor Boveri more than 100 years ago—possits that any spontaneous or exogenously induced occurrence of an unbalanced number of chromosomes (i.e., aneuploidy) precipitates an autocatalytic cascade that increases the extent of aneuploidy in each affected cell progressively, leading inevitably to eventual genomic instability and oncogenic transformation (see, e.g., Duesberg et al. 1998, Stindl 2008). Aneuploidy arises when chromosomes missegregate (i.e., fail to segregate in a numerically balanced way), either during germ-cell division (i.e., via chromosome nondisjunction during the anaphase of meiosis) or during somatic-cell division (i.e., via chromosome nondisjunction or loss during mitosis). The aneuploidy theory posits that most spontaneous non-hereditary cancer is explained by spontaneous rates of aneuploidy observed in all tissues of all higher organisms, and that chemical and other carcinogens increase cancer risk by acting, directly or indirectly, to increase the spontaneous background rate of aneuploidy. The spontaneous background rate of aneuploidy in mammalian cells is approximately 10−5 per cell division (Torres et al. 2008). This rate can be increased by exposure to ionizing radiation or to certain chemicals, either by direct damage to chromosomes, or by (e.g., chemically induced) damage to one or more proteins that make up the cellular machinery involved in meiotic or mitotic chromosome segregation, such as spindle microtubules, centromeres, kinetochores, dynein and kinesin motor proteins, the MAD2 spindle checkpoint protein, other centrosome components,
or telomeres (Kirsch-Volders et al 1996, Parry et al. 2002). Regardless of inducing agent, induced aneuploidy should have a threshold-like dose–response curve whenever the mechanism involved requires the failure of large or multiple targets associated with proper chromosome segregation (Elhajouji et al. 1995, 2011, Parry et al. 2002).

Among the most recent of the half dozen or more theories currently challenging the somatic mutation theory or tumorigenesis (Bogen 2013b; Baker 2014, 2015) is one—the Dysregulated Adaptive Hyperplasia (DAH) hypothesis (Bogen 2013b)—that posits a pivotal role for dysregulation of the entirely new paradigm of microRNA-mediated genome expression that was just discovered in the 1990 s, but later shown to exhibit a large and expanding set of diagnostic and prognostic associations with a wide variety of neoplastic progression and phenotypes (Dalmay and Edwards 2006, Hagan & Croce 2007, Pogribny 2009). In a powerful and direct test of the somatic mutation theory, the highest-resolution experimental bioassay ever done gathered data on liver and stomach tumors induced by one of the most potently mutagenic CCs known, dibenzo[a,l]pyrene (DBP), in >40,000 juvenile trout, a very well-studied biological model or tumorigenesis (Bailey et al. 2009). Dose–response patterns exhibited for both DBP-induced tumor types were shown to be remarkably nonlinear and, when interpreted together with observations that DBP can induce adducts and mutations with approximate LNT dose–response in vivo, to provide evidence inconsistent with the current regulatory default assumption that mutations drive tumorigenesis, but consistent with the DAH hypothesis (Bogen 2014a). The most recent evidence that is profoundly inconsistent with the somatic mutation theory is the remarkable observation that when induced in vivo at high rates in rat liver by aflatoxin-B1, tumors—but not potently mutagenic DNA adducts—are completely prevented in that tissue if a potent, triterpene anti-inflammatory/anti-proliferative agent is also administered (Johnson et al. 2014). Continuing, and arguably deepening, fundamental uncertainty concerning the validity of the somatic mutation theory of carcinogenesis as a pivotal basis for EPA’s default LNT risk extrapolation approach for genotoxic environmental chemical carcinogens highlights the fact that this default approach represents a health-protective policy that bridges fundamental gaps in knowledge for the practical purpose of enabling quantitative risk assessment—a policy that was never intended to produce accurate or unbiased estimates of low-level chemical carcinogen risks based on the latest science (Bogen 2014b).

5.2 Tumorigenic MOAs relevant to MTBE and its metabolites

The magnitude of potential human cancer risk posed by a defined environmental exposure to a particular chemical carcinogen is determined jointly by the magnitude and duration of exposure, the cancer potency (or increased cancer risk per unit dose) of that carcinogen, and the nature of the dose–response relationship for that carcinogen under those conditions of exposure. Since the increased cancer risk is rarely if ever observed directly (epidemiologically or experimentally) at very low levels of exposure, hypotheses concerning the pattern of dose–response that may exist at very low doses are based on a hypothesized MOA for that carcinogen, and on a hypothesis that the assumed MOA is relevant to humans (Meek et al. 2003, U.S. EPA 2005a). Thus, MOA considerations are pivotal to any assessment of MTBE cancer risk.

Most agencies evaluating possible carcinogenic risk posed by human exposure to MTBE have determined that there is insufficient evidence to determine the magnitude of any such risk posed by exposure levels that otherwise are non-toxic. For example, the IARC classified MTBE in Group 3, not classifiable as to its carcinogenicity to humans, based on limited evidence in experimental animals and inadequate evidence in humans (IARC 1999). WHO IPCS (1998) concluded that MTBE should be considered a rodent carcinogen but that it is not genotoxic, with a carcinogenic response evident only at high levels of exposure that also induce other adverse effects, and the weight of evidence supporting a conclusion that, while MTBE is a rodent carcinogen, data are insufficient to reach any conclusions about its potential to cause human cancer. More recently, McGregor (2006) concluded that evidence for carcinogenicity in rodents for MTBE and TBA is unconvincing, the strongest being for a low-level incidence of renal tubule cell adenoma increase by a mechanism that is specific to male rats and has no human relevance. Likewise, Cruzan et al. (2007) evaluated MTBE carcinogenicity based on results of animal studies, noting that a weak tumorigenic response was reported for both MTBE and TBA in one tumor type (kidney) in male rats, only for MTBE in another tumor type (testicular) in male rats, only for MTBE in one tumor type (liver) in female CD1 mice, and only for TBA in one tumor type (thyroid) in female mice. (However, as noted in Section 4.1, and as reflected in Table 3 in Section 6.2, after adjusting for mortality the liver-carcinoma response in male CD1 mice exposed chronically to MTBE by inhalation was significantly elevated.) Cruzan et al. (2007) concluded that, because the weight of evidence does not support a genotoxic MOA for these weakly tumorigenic chemicals, “It is, therefore, unlikely that humans would be exposed to sufficient levels of MTBE to cause these tumorigenic responses.” Similarly, Phillips et al. (2008) concluded:

Controlled human studies of MTBE exposure generally found no adverse health effects in the exposed study subjects. … The MTBE concentrations to which the general public is exposed are an order of magnitude lower than the recommended EPA drinking water advisory value of 20 μg/L, which is based on odor. … it may be reasonably concluded that the current guidelines for exposure reflect sound objective scientific rationale and that the vast majority of persons passively exposed to MTBE need not be concerned with serious adverse health effects.

Tumorigenic MOA considerations are discussed below only for those specific tumor types for which objective statistical evidence exists that chronic chemical exposure was associated in any study with increased tumor incidence in experimental animals chronically exposed to MTBE, or to any MTBE metabolite. MOA considerations discussed do not include those relating to any metabolite-induced tumor types for which evidence from multiple studies indicates either that no corresponding increased tumor incidence occurred after chronic MTBE exposure, or that the metabolite does not add to a systemically circulated burden because where it is produced.
it is instead effectively deactivated by further metabolism to nongenotoxic metabolites. As discussed previously, the tumor types for which such evidence exists are (1) kidney proximal tubule tumors in male rats, (2) liver tumors in male and female mice, and (3) testicular interstitial cell tumors in male rats. The likely MOA of increased thyroid tumor incidence associated with chronic exposure to TBA in rats is also discussed below. The basis for concluding that the MTBE metabolite formaldehyde does not add to a detectable, systemically circulated formaldehyde concentration, but instead is effectivel deactivated where it is produced by being further metabolized to nongenotoxic metabolites, was previously discussed.

5.2.1 Kidney

Several studies indicate that MTBE and its metabolite TBA adversely affect kidney tissue of rats exposed experimentally for prolonged periods, and do so with greater potency in male rats involving some forms of pathology observed to occur exclusively in male rats. Bermudez et al. (2012) investigated toxicity in male and female Wistar rats associated with exposure to 0, 0.5, 3, 7.5, or 15 g/L of MTBE in drinking water for 13 weeks, or to 0, 0.5, 3, 7.5 g/L (males) or to 0, 0.5, 3, or 15 g/L (females) of MTBE in drinking water for one year. Male kidney weights increased following 13 weeks, 6 months, and 1 year of exposure to MTBE concentrations of 7.5 g/L or greater, and were increased in females at concentrations of 15 g/L for 13 weeks. Hyaline droplets were detected in 0, 0, 1, 4, and 10 of a total of 10 male rats per dose group examined for this endpoint among rats exposed to 0, 0.5, 3, 7.5, and 15 g/L MTBE, respectively, in drinking water for 13 weeks. No hyaline droplets were detected in kidneys of any of the female rats examined after 13 weeks of exposure, based on examining 10 rats of each sex in each dose group. Tubular epithelial cell replication, measured as significantly elevated BrdU labeling index, occurred in 0, 2, 3, 3, and 6 of the 10 male rats examined in each exposure group, but in only three of the 50 female rats examined in all exposure groups. These data reported by Bermudez et al. (2012) indicate statistically significant positive dose–response trends for the incidence of hyaline droplets ($p = -0$) as well as tubular epithelial regeneration ($p = 0.0045$), by applying the corresponding exact two-tailed Cochran–Armitage trend tests to those data. Dodd et al. (2013) extended the one-year study design used by Bermudez et al. (2012) out to an exposure period of two years. The most sensitive adverse endpoint identified among all those associated with chronic, two-year exposure to MTBE via drinking water was significantly increased kidney weight in all concentration groups of male and female Wistar rats (Dodd et al. 2013). The lowest concentration tested in that study (0.5 g/L) corresponded to nominal average ($\pm 1$ SD) intakes of 25 $\pm 11$ and 49 $\pm 14$ mg/kg/day for male and female rats, respectively (Dodd et al. 2013).

A male-rat-specific alpha2 u-globulin-mediated carcinogenic MOA for certain xenobiotic chemicals (including nongenotoxic hydrocarbons such as $d$-limonene, unleaded gasoline, 1,4-dichlorobenzene, and 2,4,4-trimethylpentane) has long been well recognized to involve chemically induced renal neoplasms in male rats resulting from chemical binding to alpha2u-globulin (one of the most abundantly synthesized low-molecular-weight proteins synthesized in male rat liver), lysosomal accumulation of this bound protein (forming “hyaline droplets” that exhibit considerably enhanced visualization after Mallory–Heidenhain staining), consequent nephropathy and cell death, and compensatory cell proliferation as components of a carcinogenic MOA that is widely recognized as not being relevant to predicting cancer risk to humans (U.S. EPA 1991, Swenberg 1993, Hard et al. 1999, IARC 1999, Doi et al. 2007). Histopathology evidence supports the current scientific consensus that this male-rat-specific MOA is at least the predominant cause of renal tubule tumors in male rats exposed chronically to TBA, which is nongenotoxic (McGregor 2010, see Section 3.3), is the major metabolite of MTBE (see Section 2.1.3), selectively binds to alpha2u-globulin only in male rats, and induces kidney proximal tubule tumors only in male rats by the recognized alpha2u-globulin MOA (NTP 1995, Borghoff et al. 2001, McGregor and Hard 2001, Borghoff et al. 2001, NSF International 2003, API 2005, McGregor 2010, Hard et al. 2011). Specifically, in male but not female rats exposed to TBA, protein droplets accumulate in kidney tubules (Borghini et al. 2001), and TBA exposure causes accumulation of angular protein-droplet precursors of outer-medulla granular casts and linear papillary mineralization that are typical of alpha2u-globulin nephropathy in treated male rats (Hard et al. 2011). Among all rats exposed subchronically to 0, 0.25, 0.5, 1, 2, or 4% (w/v), TBA administered in drinking water to male F344 rats for 13 weeks, there were large reductions in urine volume in the three highest dose groups with crystaluria occurring in the two highest dose groups, nearly all exposed rats had increased bile acids, and proliferating cell nuclear antigen or PCNA-stained S-phase nuclei (indicative of cell replication) in renal sections were significantly increased in male rats in the 1% and 2% TBA exposure groups (Lindamood et al. 1992). After these concentrations of TBA in drinking water were administered to male F344 rats for 90 days, hyaline droplets accumulated in renal proximal tubules with crystaline, rectangular, and rhomboid forms of the protein evident, and associated nephropathy, that increased in a dose-related pattern, except (evidently due to increased mortality) in the 4% dose group, and replicative DNA synthesis as measured by immunohistochemical staining for proliferating cell nuclear antigen was increased in proximal tubules of rats dosed with 2% TBA (Takahashi et al. 1993). In vivo interaction of relatively nonvolatile TBA with alpha2u-globulin was observed in experiments in which male and female F344 rats were given a single gavage $^{14}$C-TBA dose of 500 mg/kg (Williams and Borghoff 2001). Concentrations of radiolabel found in renal cytosol 12 h after dosing were higher in males than in females, and both gel filtration and ion-exchange chromatography showed radiolabel detected in male rat renal cytosol co-eluted with alpha2u-globulin (Williams and Borghoff 2001). The radiolabel in renal cytosol of rats dosed with radiolabeled TBA could be displaced from the low-molecular-weight protein fraction of male, but not female, rat kidney by $d$-limonene oxide (Williams and Borghoff 2001), which has a particularly high affinity to alpha2u-globulin (Lehman-McKeeman et al. 1989). Exposure to TBA was very clearly associated with dose-related, statistically significant increases in mitotic DNA synthesis labeling index measured using BrdU in epithelial cells of the renal cortex of
male but not in female rats exposed to TBA in air for 10 days at concentrations of 250–1,750 ppm (Borghoff et al. 2001).

In the two-year NTP drinking water study of TBA, hyperplasia was observed in 14/50, 20/50, 17/50, and 25/50 male rats of the 0, 1.25, 2.5, and 5.0 mg/mL drinking water dose groups, respectively (p < 0.01), while renal tubule hyperplasia developed only in a single rat in the highest dose group among females administered up to 10 mg/mL of TBA in water (Cirvello et al. 1995, NTP 1995). As a subsidiary contributing nongenotoxic MOA, chronic TBA exposure may also act to elevate renal tubule tumor incidence observed in male rats by exacerbating CPN, a spontaneous age-related disease that occurs with high incidence (particularly among males) in strains of rat commonly used for subchronic and chronic toxicity studies, and that (like the male rat alpha2u-globulin MOA) has no human counterpart and is not considered relevant for purposes of characterizing human risk (McGregor 2010, Hard et al. 2011, 2013). Both alpha2u-globulin nephropathy (involving cell loss through lysosomal overload) and CPN (involving a spontaneous disease process with a high rate of cell turnover) represent secondary events and not direct forms of cytotoxicity, and other than these two processes that have no human counterpart, there is no evidence of TBA-induced renal cytotoxicity in either male or female rats (Hard et al. 2011).

Bird et al. (1997) did not, on an individual animal basis, address the correlation of end-stage CPN with renal tumors they observed in male rats chronically exposed by inhalation of 3,000 ppm MTBE, so CPN as an ancillary MOA for those renal tumors was not established directly. Rather, contributing role of MTBE-exacerbated CPN in elevating renal tumor incidence in male rats exposed chronically to 3,000 ppm MTBE is supported by indirect evidence. CPN was reported by Bird et al. (1997) in all treated animals, but the greatest severity was observed in the highest two exposure groups (particularly so in male rats), and exposure-related decreases in male rat survival were attributed primarily to CPN (Section 4.1). In this respect, MTBE appears to enhance or accelerate the pattern of spontaneous CPN that is observed in aging rats (Hard et al. 1999).

CPN has enhanced proximal tubule apoptosis and regenerative hyperplasia as a characteristic in common with the male-rat-specific pattern of alpha2u-globulin nephropathy that is induced together with low renal tumor incidence by chronic exposure to TBA and other light hydrocarbons (Hard et al. 1999, see also the discussion of CPN above this subsection). MTBE generates TBA as its major, persistent metabolite, and like TBA, MTBE induces kidney tubule tumors in exposed male but not female rats (McGregor 2006, Leavens and Borghoff 2009). Like TBA, MTBE binds to alpha2u-globulin in the kidney of male F344 rats as measured by headspace concentrations of MTBE in kidney samples from rats exposed orally to MTBE (Prescott-Mathews et al. 1999), either with or without the addition of d-limonene-1,2-oxide, a metabolite of d-limonene that is also known to bind alpha2u-globulin (Lehman-McKeeman et al. 1989). The presence of d-limonene-1,2-oxide resulted in higher headspace concentrations of MTBE at 4 and 12 h after administration of radiolabeled MTBE, while this effect was not seen in kidneys of female rats similarly exposed to radiolabeled MTBE (Prescott-Mathews et al. 1999). There was also an increase in the measured alpha2u-globulin concentration in the kidneys of male F344 rats that was significant at the highest exposure concentration, 1750 ppm, and that also correlated with increased renal cell proliferation (Prescott-Mathews et al. 1999). The measured binding affinity of MTBE to alpha2u-globulin (Poet and Borghoff 1997) is much lower than that of other known binders such as 2,4,4-trimethyl-2-pentanol (Borghoff and Lagarde 1993) and d-limonene oxide (Lehman-McKeeman et al. 1989), but is similar to that of 1,4-dichlorobenzene, a chemical that also binds alpha2u-globulin and induces associated nephropathy (Charbonneau et al. 1989). BrdU labeling index was increased in the renal cortex of all MTBE-treated male groups (lowest dose 400 ppm) and in the outer part of the medulla in the two highest male exposure groups of F344 rats exposed to MTBE for 10 days (Prescott-Mathews et al. 1997). After male and female F344 rats were exposed to MTBE vapors of 0, 413, 1516, or 3013 ppm for 6 h/day for 10 consecutive days, there were significant proximal tubule necrosis and protein-droplet accumulation in the two highest exposed groups of male rats, a significantly increased amount of kidney alpha2u-globulin in male rats exposed to 3013 ppm, significantly greater cell proliferation labeling indices in all the exposed groups of male rats, and a strong positive correlation (r = 0.994) between the observed exposure-related levels of enhanced kidney proximal-tubular-cell proliferation and alpha2u-globulin concentration; no similar responses were observed in female rats (Prescott-Mathews et al. 1997). After exposure of F344 rats to 0, 400, 3,000, or 8,000 ppm of MTBE for 5 or 28 days, increased protein accumulation was observed in Mallory–Heidenhain-stained kidney tubular epithelial cells of male rats in the two highest exposure groups, and those cells in the male (but not female) rats exhibited significantly enhanced rates of cell proliferation labeling in the two lower exposure groups by day 5, and in all exposure groups by day 31 (Bird et al. 1997). In their corresponding two-year cancer bioassay of F344 rats exposed to MTBE concentrations up to 8,000 ppm, kidney tumors were elevated only in male rats that were exposed to 3,000 ppm MTBE in air (Section 4.1).

The similarity of kidney pathologies observed in male rats after exposure to TBA and to MTBE suggests the possibility that enhanced nephropathy induced by MTBE in male rats, involving dose-related enhanced turnover of proximal tubule epithelial cells, is caused at least in part by (ATSDR 1996, p. 136), and perhaps directly and solely by, the TBA that is produced in a one-to-one ratio for each molecule of MTBE metabolized. In view of currently applied mathematical models of mammalian tumorigenesis, any cytotoxic exposure scenario that leads to tissue-specific aging involving accelerated apoptosis and regenerative hyperplasia is expected to increase tumor likelihood in that tissue via a nonlinear tumor-promotion MOA (see Supplementary Appendix C to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367).

Alternatively, the similarity could simply be what might be expected of two weakly active compounds (MTBE and TBA) acting independently via the same male-rat-specific alpha2u-globulin MOA (McGregor 2006).

A third alternative is that renal tubule tumors induced by MTBE exposure are due entirely or in part to hypothesized genotoxicity exerted in vivo by the MTBE metabolite form-
aldehyde, which is formed in addition and in equal amounts to the nongenotoxic TBA metabolite (i.e., again in a one-to-one ratio for each molecule of MTBE metabolized). However, this third hypothesis begs the question of why MTBE-induced kidney tumors occur only in male rats, in view of the fact that the yield of plausibly genotoxic formaldehyde from MTBE (although such genotoxicity is speculative—see Section 3.2) is expected to be roughly equal in male and female rats and is generated in both sexes as a metabolite of systemically distributed MTBE. The genotoxic MOA hypothesis also presumes the carcinogenic potency of MTBE to induce renal tubule tumors should be far greater than that of TBA alone, insofar as this hypothesis posits that an additional site-specific carcinogenic species (namely, formaldehyde) is generated in addition to TBA from each molecule of MTBE that is metabolized. However, this presumption appears to be contradicted by the fact that the estimated upper-bound potency of retained TBA derived from the retained portion of administered MTBE to induce renal tubule tumors (0.00092 or 0.0037 per mg TBA-equivalent dose/kg/day, based on potencies estimated using bioassay data from MTBE-inhalation studies by Bird et al. 1997 and Benson et al. 2011, respectively) is 1.3- or 5.1-fold less than that of the retained portion of administered TBA itself (0.0047 per mg TBA/kg/day, estimated from NTP 1995 bioassay data—see Table 4 in Section 6.2). As discussed in Section 4.1, the F344 rats studied by Benson et al. (2011) were not administered MTBE alone, but rather were administered GVC with or without MTBE.

To enable the comparison TBA to MTBE potency just described, TBA-equivalent doses and potency using data from the Benson et al. (2011) study were estimated by applying PBPK relationships relating retained to administered dose for MTBE and for TBA that are shown in Figures 2 and 6 (Section 2.2), respectively (see footnote f of Table 4). A source of uncertainty affecting the TBA-equivalent potency estimate obtained for the Benson et al. (2011) study is that GVP compounds besides MTBE may have affected values of parameters of the Leavens and Borghoff (2009) PBPK model of MTBE (alone, without GVC) and TBA in rats that was used to generate predictions from which the relationships shown in Figures 2 and 6 were estimated. This source of uncertainty is not treated quantitatively in the present study, and it is unknown whether or how it may bias the TBA-equivalent potency estimate obtained for the Benson et al. (2011) study. However, this source of uncertainty does not affect the TBA-equivalent potency estimate obtained for the Bird et al. (1997) study.

The available experimental tumor data are therefore inconsistent with the hypothesis that a reactive MTBE metabolite, such as formaldehyde, participates in any way to augment renal tumorigenicity of the TBA that is generated by MTBE metabolism. That is, based on quantitative potency considerations, the renal tumorigenic potency of MTBE in male rats is explained (quantitatively) entirely by that of the TBA generated by MTBE metabolism. In view of this observation, the weight of evidence reviewed here supports the conclusion that elevated rates of incidence of renal proximal tubule tumors in male F344 rats exposed to ≥3,000 ppm of MTBE by inhalation for 6 h/day, 5 days/week, for ≥84 weeks (Bird et al. 1997), and in male F344 rats exposed to ≥555 ppm MTBE (plus GVC, at total vapor concentrations ≥10 g/m³) by inhalation for 6 h/day, five days/week, for two years (Benson et al. 2011), were very likely caused by a nongenotoxic MOA mediated by MTBE-induced enhancement or acceleration of an age-related pattern of combined CPN, proximate protein accumulation in kidney tubule, and associated epithelial cell turnover, to which male rats are uniquely susceptible. The same, or an overlapping, MOA widely recognized to account for elevated rates of incidence of renal proximal tubule tumors in male F344 rats exposed to the MTBE metabolite, TBA, is well recognized to be irrelevant to humans.

Melnick et al. (2012) challenged the hypothesis that enhanced CPN is an MOA for chemically induced kidney cancers with an analysis of NTP bioassay tumor data indicating that chemically exacerbated CPN is not associated with, and by extension not the cause of, renal tubular tumor induction in rats. While this study showed that the mean CPN severity did not correlate with renal tubule tumorigenicity, their analysis of CPN severity and renal tubule tumors only compared aggregated tumor data, and was not based on analysis of data pertaining to tumors in individual rats. In contrast, CPN of advanced severity is strongly and positively associated with renal tumor the development (Hard et al. 2013). A reevaluation of archived kidney sections obtained from NTP studies in which CPN was graded for from animals treated with one of 20 compounds reported that advanced stages of CPN represent a risk factor for the development of renal tumors (Hard et al. 2012). The difference in CPN severity between males and females was also observed to correlate well with tumor outcomes. Thus, CPN severity observed in studies of MTBE and TBA probably contributed to exposure-related elevations of renal tumor incidence in those studies.

5.2.2 TBA-Related Thyroid Tumors

Groups of 60 male and 60 female B6C3F1 mice were exposed for two years to drinking water containing TBA concentrations of 0, 5, 10, or 20 mg/mL—that is, 0, 5,000, 10,000, or 20,000 ppm—corresponding to approximate lifetime TWA doses of 0, 540, 1,040, or 2,070 mg/kg/day to males, and 510, 1,020, or 2,110 mg/kg/day to females (NTP 1995; Cirvello et al. 1995). Results from this study provided “equivocal evidence of carcinogenic activity of TBA alcohol in male B6C3F1, mice based on the marginally increased incidences of follicular cell adenoma or carcinoma (combined) of the thyroid gland,” and “some evidence of carcinogenic activity of TBA alcohol in female B6C3F1 mice based on the marginally increased incidences of follicular cell adenoma of the thyroid gland” (NTP 1995). The greatest increase in the incidence of follicular cell adenoma or carcinoma in male mice occurred in the 10-mg/mL group of male mice, and this increase was not statistically significant (two-tailed \( p = 0.20 \), by Fisher’s exact test). The only increase in follicular cell adenoma incidence in female mice, which occurred in the highest (20-mg/mL) group, was not statistically significant by two-tailed Fisher’s exact test \( p = 0.053 \), but the set of all female mouse data for this endpoint was reported as showing a significant \( p = 0.039 \) positive trend using the Dins–Haseman logistic regression test with an unspecified number of tails (NTP 1995, Table 14, p. 53), and also shows a significant \( p = 0.010 \) positive trend using the two-tailed exact Cochran–Armitage trend test (Agresti 2002).
In contrast, the study conducted by Burleigh-Fayer et al. (1992) exposed CD-1 mice by inhalation to MTBE, and found no increase in the incidence of thyroid follicular cell tumors. Importantly, the effective plasma level of TBA following inhalation exposure to MTBE would likely be higher than the plasma concentration following exposure via drinking water as in the NTP study (1995). This may suggest strain differences between the mice tested.

A nongenotoxic MOA for significantly increased thyroid tumors in female mice is indicated by evidence supporting the conclusion that TBA is nongenotoxic to mammalian cells in vivo. A nongenotoxic MOA for these tumors is also supported directly by evidence that TBA simulates thyroid follicular cell proliferation, and is thereby expected to amplify spontaneous thyroid follicular cell tumors when administered at doses that are patently mitogenic. The incidences of thyroid follicular cell hyperplasia were significantly increased in all groups of TBA-exposed males, and in the two highest groups of TBA-exposed females (NTP 1995). Among the nine high-dose-group female mice who exhibited thyroid follicular cell adenoma, six also exhibited thyroid follicular cell hyperplasia (McGregor 2010). Hyperplastic follicular cell lesions that occurred in the TBA-treated mice were similar in appearance to those that occurred in untreated mice (NTP 1995), which supports the hypothesis that TBA “promotes” or accelerates spontaneous hyperplasia that normally occurs in this mouse tissue. Although there is some evidence that TBA might stimulate thyroid cell growth indirectly through its demonstrated ability in B6C3F1 mice to alter circulating levels of hormones that tightly control thyroid growth and change liver-enzyme activities that can affect circulating thyroid hormone levels (Blanck et al. 2010), this experimental evidence does not yet clearly support a defined etiologic hypothesis, so the specific nongenotoxic MOA of observed increases in both hyperplasia and adenomas involving female mouse thyroid follicular cells remains unclear (McGregor 2010). The likelihood that this MOA is nongenotoxic, however, is supported by the lack of evidence for TBA-induced genotoxicity. If it were true that TBA induced thyroid tumors in mice by a genotoxic MOA, the same or an even greater response would be expected after chronic MTBE exposure, because relatively nonvolatile TBA is excreted relatively slowly from systemic circulation and so readily partitions into thyroid and other body tissues, and nearly every molecule of retained MTBE is metabolized not only to one molecule of TBA, but also to one molecule of the genotoxic (nasal/nasopharyngeal) carcinogen formaldehyde. In fact, contrary to this expectation, none of the multiple rodent bioassays done to assess the carcinogenic potential of MTBE has indicated that chronic MTBE exposure can significantly increase the incidence of thyroid follicular cell tumors. Consequently, the relevance of elevated thyroid follicular cell adenomas in a single study of mice exposed to the highest drinking water concentration of TBA investigated, to human cancer risk assessment, is unclear. Since the only other tumor type known to be significantly elevated after chronic exposure to TBA (male rat proximal renal tubule tumors associated with enhanced C-type diuretic peptide or CNP and alpha2u-globulin accumulation) has a MOA irrelevant to humans, the existing set of cancer bioassay data for TBA is inadequate to assess the potential human carcinogenicity of this MTBE metabolite.

5.2.3 Liver
In CD-1 mice exposed to 0, 400, 3,000 or 8,000 ppm MTBE by inhalation for 6 h/day, five days/week for 18 months, incidences of hepatocellular adenomas, and of combined hepatocellular adenomas and carcinomas, were statistically significantly increased in female mice exposed to 8000 ppm of MTBE; hepatocellular tumors were significantly increased in male mice in the highest exposure group after adjusting for mortality (Bird et al. 1997, CalEPA OEHHA 1999, Table 9 on p. 58; see also Section 4.1). However, mortality was increased and the mean survival time was decreased in the high-dose male mice compared with controls. Body weight gain was also decreased in the 8,000-ppm group compared with the controls (a decrease of 16% and 24% for male and female mice, respectively), indicating that the high dose exceeded the MTD in this study (Bird et al. 1997). In particular, relative kidney weights were significantly elevated in all male MTBE exposure groups and in the 8000-ppm female mice, and liver weights were increased significantly in the two highest exposure groups of female mice (Bird et al. 1997). Since MTBE is generally negative in mutagenicity tests, and the hepatocellular tumors induced by MTBE in CD-1 mice were detected only in the high-dose animals where the dose exceeded the MTD, Burleigh-Flayer et al. (1992) and Bird et al. (1997) considered the mouse liver tumor result in their study not likely to be due to a direct DNA-acting phenomenon.

Twelve or thirty-two weeks of inhalation exposure to ~8,000 ppm of MTBE did not result in liver-tumor-promoting activity after 12-day-old B6C3F1 female mice were first administered a powerful tumor-initiating agent, N-diethylnitrosamine (DEN), at 5 mg per kg BW (Moser et al. 1996b). This subchronic MTBE exposure significantly increased liver weight and hepatic microsomal CYP enzyme activity; however, no signs were observed of hepatotoxicity, any increase in proliferative DNA synthesis as measured by BrdU incorporation, or any significant increase in the mean size of hepatic foci or volume fraction of the liver occupied by foci as compared with DEN-initiated controls at either 16 or 32 weeks. By comparison, in short-term studies in mice performed by the same research team at the Chemical Industry Institute for Toxicology or CIIT, female B6C3F1 mice were exposed by inhalation to 7,814 ppm of MTBE vapor for 6 h/day five days/week for three or 21 days. Compared with unexposed mice, the MTBE-exposed mice had increased relative liver weight, increased CYP enzyme content and activity, and decreased relative uterus weight. Hepatic labeling index as measured by BrdU incorporation into DNA was increased in all exposed mice at 3 days but not 21 days, indicating that MTBE and 91-01 unleaded gasoline are also hepatic mitogens. In a parallel study, gavage treatment of female B6C3F1 mice with 1,800 mg of MTBE per kg BW per day for three days resulted in increased estrogen metabolism in isolated mouse hepatocytes, consistent with the authors’ conclusion that increased in hepatic estrogen metabolism and uterine weight indicates a potential role for endocrine modulations in MTBE-induced hepatocarcinogenesis (Moser et al. 1996a,b). An NRC panel considering MTBE health risks concurred that “nongenotoxic hormonally related mechanisms are the most plausible explanation” for the development of liver tumors in female mice chronically exposed to MTBE by
inhalation, concluding also that while these tumor data are the “most reliable data available for risk-assessment purposes … it should be recognized that this amounts to extremely weak evidence of carcinogenicity … [that] cannot be discounted” (NRC 1996).

In a follow-up study, Moser et al. (1998) exposed female B6C3F1 mice by inhalation to 8,000 ppm of MTBE vapor for three or 21 days or for four or eight months under conditions similar to the Bird et al. (1997) two-year MTBE bioassay. MTBE exposure significantly decreased BW gain and ovary and pituitary weight at four and eight months and uterine weight at all time points (Moser et al. 1998). After eight months of MTBE exposure, the mean number of days in both the estrus and nonestrus stages of the estrus cycle was significantly increased, and histological evaluation of hematoxylin and eosin-stained tissues showed a decrease in the number of uterine glands (Moser et al. 1998). Proliferative DNA synthesis as measured by BrdU incorporation was decreased in uterine glandular and luminal epithelial cells, and the number of epithelial layers in the cervix and vagina was decreased, after each MTBE exposure duration examined; however, MTBE did not bind competitively to estrogen receptors, and did not alter serum estrogen levels or alter the location or intensity of estrogen receptor immunoreactivity in the uterus, cervix, and vagina, indicating that while MTBE exposure causes multiple endocrine-related tissue and cellular responses, none are evidently mediated by the estrogen receptor (Moser et al. 1998).

MTBE is metabolized by CYP to formaldehyde, a potentially mutagenic intermediate capable of binding covalently to DNA, RNA, and protein. As discussed in that section of this report, Casanova and Heck (1997) showed that

- *In vitro* yields of protein–DNA–formaldehyde crosslinks and RNA–formaldehyde adducts isolated from hepatocytes from female CD-1 mice incubated with 14C-radiolabeled MTBE were very small and were independent of the MTBE concentration in the hepatocyte suspension over a wide (0.33–6.75-mM) concentration range.
- Similar results occur when using hepatocytes from either male B6C3F1, mice or male F344 rats.
- Prior induction of CYP failed to increase these yields.

In contrast, the formaldehyde-related damage yields in that study increased markedly in proportion to the concentration of 14C-formaldehyde added directly to the medium (Figure 7). A similar, approximately linear concentration–response relationship was observed for formaldehyde-induced DNA damage in relation to the concentration in which male mouse B6C3F1 hepatocytes (but not rat, or hamster, or human hepatocytes) were incubated with methylene chloride (dichloromethane) (Andersen et al. 1980). As noted previously, like MTBE, dichloromethane is a small molecule that is metabolized in mouse liver to formaldehyde, but in this case the metabolic transformation is catalyzed primarily by the enzyme glutathione-S-transferase that exhibits little if any saturation in reaction rate at high dichloromethane concentrations (Andersen et al. 1980). In contrast, metabolism of MTBE to formaldehyde by CYP oxidase was saturated at concentrations well below those required to induce liver neoplasms in female CD-1 mice.

Similarly, no increase in control endogenous levels of formaldehyde-induced protein–DNA crosslinks were detected after administering either methanol (1 g/kg BW, by gavage) or aminopyrine—two small molecules that each are readily metabolized in liver to formaldehyde (see Section 2.1.6)—to male Sprague-Dawley rats, with or without co-administration of 0.6 g/kg of the acetaldehyde oxidase inhibitor disulfiram that is expected to reduce the rate of formaldehyde metabolism and thereby tend to maximize attainable hepatocellular formaldehyde concentrations *in vivo* (Lutz 1986a). The lack of concentration dependence, the absence of species or sex differences in the formation of MTBE-associated DNA or RNA damage, and the lack of such detectable damage in rat hepatocytes after *in vivo* administration of similar small molecules that are readily metabolized to formaldehyde, provide evidence that formaldehyde plays no critical role in the MOA for MTBE-induced liver tumors in female CD-1 mice. Casanova and Heck (1997) concluded from this that, at least for P450-metabolized chemicals such as MTBE, methanol, and aminopyrine, the toxicity of metabolically generated formaldehyde “is expressed only in the organ in which it is produced, and only when the rate of its production is sufficiently high that normal cellular defense mechanisms are overwhelmed.” Likewise, juvenile male BALB/c mice exposed for 51 days to 0, 80, 800, or 8,000 ppm of MTBE in drinking water showed no evidence of dose-related effect on any of three measures made of hepatic oxidative stress: MDA, Trolox-equivalent antioxidant capacity, and 8-hydroxy-2′-deoxyguanosine DNA adducts (de Peyster et al. 2008).

With regard to significantly higher hepatocellular adenoma incidence in CD-1 mice exposed to 8,000 ppm in the study reported by Burleigh-Flayer et al. (1992) and Bird et al. (1997), McGregor (2006) emphasized that because there is no supporting evidence of similar effects in rats of any of the other studies considered relevant to an evaluation of MTBE carcinogenicity, the scientific basis for an assumption that metabolically generated formaldehyde explains MTBE-related mouse liver tumors is “extremely weak and is not an adequate basis for considering liver as a target for neoplastic effects of any human significance.” However, such a tumorigenic MOA is theoretically consistent with the (albeit limited) evidence for increased rates of lung tumors in male F344 rats and pheochromocytomas in female F344 rats that for two years inhaled air containing up to 1,000 ppm of methanol (Section 4.4). Although the parallel study using similarly exposed male and female B6C3F1 mice showed no clear evidence of tumorigenicity, including in the liver (Section 4.4), the absence of elevated liver tumors in that study could be due to the fact that its highest methanol exposure level (1,000 ppm, or 0.0409 mmol/L) was 8-fold less than the 8,000-ppm (or 0.328-mmol/L) concentration of MTBE shown by Bird et al. (1997) to elevate mouse liver tumor incidence. However, a formaldehyde-MOA interpretation of methanol-induced lung tumors in male F344 rats is not consistent with the fact that lung tumors are not among tumor types observed to be elevated in rodents chronically exposed to MTBE by any route.

The weight of available evidence reviewed here, and in Section 4, supports the conclusion that elevated liver tumor incidence in female CD-1 mice exposed to 8,000 ppm of MTBE by inhalation for 6 h/day, five days/week, for 18 months...
observed in the study by Bird et al. (1997) was very likely caused by a nongenotoxic MOA mediated by dose-related hormone disruption. Hormone disruption represents an indirect MOA for tumorigenesis because first sufficient disruption must occur beyond a normally regulated, hormonally mediated process, therefore implying a threshold dose.

5.2.4 Testes

No testicular pathology was reported in a 2-year exposure in F344 rats treated via drinking water with TBA (NTP 1995), and no significant testicular changes related to treatment have been reported in CD-1 or B6C3F1 mice exposed to MTBE or TBA, respectively (NTP 1995, Burleigh-Flayer et al. 1992, Bird et al. 1997). However, increased testicular interstitial cell hyperplasia, testicular mineralization, and Leydig interstitial cell adenomas were recorded in a two-year inhalation study with MTBE in F344 rats (Chun et al. 1992, Table 39 at p. 169) and in a study of Sprague-Dawley rats exposed by gavage for two years to 0, 250, or 1000 mg/kg day of MTBE in olive oil (Belpoggi et al. 1998) (see Section 4.1). Regarding increased Leydig interstitial cell testicular tumors seen in male F344 rats exposed to 3000 or 8000 ppm of MTBE by inhalation, Chun et al. (1992) suggested that the observed “increase in the frequency of interstitial cell adenomas of testes may be a reflection of altered pituitary function [unrelated to MTBE exposure], since a larger average size of the pituitary and adenomas was observed in the control and low concentration groups.” Additionally, it is possible that because of the high incidence of CPN in the two highest dose groups, and the corresponding decreases in mean survival time, the occurrence of Leydig cell interstitial tumors studied by Chun et al. (1992) appears artifically increased in the highest two dose groups. Furthermore, in the presence of such high background occurrence in the aging rat, it is problematic to assign toxicological significance to the LCT findings. Concerning the increased testicular tumors observed in this study, Bird et al. (1997) concluded

This tumor is typically the most frequently observed spontaneous tumor in aged male Fischer 344 rats. ... The incidence observed in the MTBE-exposed groups were within the range (64–98%) previously reported for aged male Fischer 344 rats. ... The frequency of these testicular adenomas in control animals (64%) from this study was lower than that normally reported, and below the laboratory’s previous control values of 86% and 91%. Thus, the increased incidences of testicular tumors in the 3000 and 8000 ppm groups reflect the lower incidence in the control group and may not be treatment related. If the apparent dose-related incidence is a real finding, it may not necessarily be due to a direct action of MTBE but may be indirect, e.g. hormonal mediated.

The rates of testicular hyperplasia incidence in all examination sets combined (including sacrifices at weeks 82, 97, and 104, and in animals found dead or sacrificed when found moribund) in each of the 0-, 400-, 3000-, and 8000-ppm groups of male rats, respectively, were 21/50, 20/50, 20/50, and 43/50 (p < 10^-5 by Fisher’s exact test for two-tailed comparison of control versus high-dose rates, two-tailed exact Cochran–Armitage P_trend = 0.00010), and the corresponding increased incidences of seminiferous tubule mineralization were 17/50, 21/50, 29/50, and 33/50 (two-tailed exact Cochran–Armitage P_trend = 0.00038). In a study in Sprague-Dawley rats treated by gavage four days/week with MTBE for two years, while testicular interstitial cell hyperplasia (focal and diffuse combined) increased at rates of 4/60, 8/60, and 9/60 (i.e., without a significant trend) in rats dosed at 0, 250, and 1000 mg/kg/day MTBE, respectively, rates of testicular mineralization increased in a significant dose-related pattern at rates of 8/60, 12/60, and 21/60, respectively (two-tailed exact Cochran–Armitage P_trend = 0.0041) (Belpoggi et al. 1998). Testicular tubular mineralization is a regressive lesion associated with concurrent necrosis (Hamir et al. 1992), and with hyperplasia (as well as testicular interstitial cell tumors) induced with highly nonlinear dose–response in Noble (NBL/Cr) rats exposed to the metal cadmium (Waalkes et al. 1999).

In nine-week-old male Sprague-Dawley rats administered MTBE by gavage at 0, 250, 500, 1000, or 1500 mg of MTBE/kg/day for 15 or 28 consecutive days and then sacrificed 1 h following the last dose (Williams et al. 2000), compared with control animals: relative testicular weight increased significantly only in the rats dosed with 1500 mg of MTBE per kg BW for 28 days; serum triiodothyronine (T3) was significantly decreased at this time point in that dose group and in the rats exposed to 1000 mg of MTBE/kg/day; a decrease in serum luteinizing hormone (LH) and dihydrotestosterone was observed at 1500 mg of MTBE/kg/day; interstitial fluid and serum testosterone levels as well as serum prolactin levels were decreased in animals treated with 1500 mg of MTBE/kg/day for 15 days; and no testicular lesions were observed at any dose level. Williams and Borghoff (2000) investigated the hypothesis that the MTBE-induced decrease in serum testosterone levels in male Sprague-Dawley rats observed by Williams et al. (2000) may be due in part to the ability of MTBE to induce the metabolism of endogenous testosterone and hence enhance its clearance, using the same species and age of rats and same exposure route, levels, and durations as in that previous study. Total hepatic microsomal CYP enzyme levels were increased in treated male Sprague-Dawley rats as follows: 2.0-, 6.5- and 2.9-fold increases in CYP2B1/2 occurred in rats treated with 1000 mg of MTBE/kg/day for 28 days, and with 1500 mg of MTBE/kg/day for 15 and 28 days, respectively; CYP1A1/2, CYP2A1, and CYP2E1 activities were increased 1.5-, 2.4-, and 2.3-fold, respectively, in the high-dose 15-day-treated rats; CYP2E1 was increased 2.0-fold in high-dose 28-day-treated rats (2.0-fold); CYP3A1/2 was increased 2.1-fold and uridine diphosphate or UDP-glucuronosyltransferase activity 1.7-fold in high-dose 28-day-treated rats; and MTBE also induced its own metabolism 2.1-fold in high-dose 28-day-treated rats. These results indicate that MTBE induces selected enzymes involved in testosterone metabolism. Williams and Borghoff (2000) hypothesized that decreased serum testosterone observed following MTBE administration may be due to enhanced testosterone metabolism and subsequent clearance, but could not explain how this and other ways in which the male rat endocrine system is perturbed following MTBE treatment might increase the incidence of Leydig interstitial cell tumors.

de Peyster et al. (2003) investigated early endocrine changes consistent with known mechanisms of Leydig cell carcinogenesis by gavaging adult male Sprague-Dawley rats with MTBE in five different subchronic experiments addressing
testosterone biosynthesis in isolated rat Leydig cells exposed in vitro to MTBE or to its major metabolite, TBA. In vitro testosterone production declined 29–50% in Leydig cells following 3-h exposures to 50–100 mM MTBE or TBA. Within hours after gavaging with 1000 or 1500 mg/kg of MTBE, circulating testosterone declined to 38–49% of control levels (p < 0.05) and after 28 days liver P450 enzymes were elevated in both groups but significantly so (p < 0.05) in the high-dose group. After 28 days, rats dosed with 800 mg/kg/day had increased organ:BW ratios for both the adrenal and thyroid glands and increased corticosterone levels. Noting that thyroid hormones regulate both Leydig cell steroidogenesis and the expression of liver P450 enzymes involved in steroid catabolism, de Peyster et al. (2003) concluded that while results from their subchronic dosing and in vitro experiments do not fully explain the occurrence of LCTs observed in rat cancer bioassays using high chronic doses of MTBE far exceeding typical human exposures, they did identify repeated testosterone synthesis inhibition as a likely MOA for this tumor induction.

In male Sprague-Dawley rats administered 0, 400, 800, or 1600 mg/kg/day of MTBE by gavage for two or four weeks, among other observed effects, the testicular fraction of BW was reduced significantly (p < 0.05) after two weeks of exposure in all MTBE dose groups, this fraction for the thymus and for the prostate were each reduced significantly (p < 0.05) among high-dose group rats exposed for four weeks, and urinary creatinine was significantly reduced (p < 0.05 or p < 0.01) in all groups of rats exposed to MTBE for two or four weeks (Dong-mei et al. 2009). Another study in which 0, 400, 800, or 1600 mg/kg/day of MTBE was administered by gavage to male Sprague-Dawley rats for 30 consecutive days reported a decreasing trend for LH and testosterone in experimental groups (r = −0.50 and p = 0.030, and r = −0.67 and p = 0.002, respectively) (Khalili et al. 2015). The effect of MTBE exposure on LH or testosterone levels has not been studied in F344 rats, so currently there is no direct evidence that such effects could have played a role in elevating Leydig interstitial cell tumors observed in F344 rats exposed chronically to MTBE.

Leydig cell adenomas are the most common tumor type in rats, and their significance to low-dose cancer-risk extrapolation for humans has been challenged (Prentice and Miekle 1995, Cook et al. 1999). Laboratory control values for the occurrence of Leydig testicular tumors in F344 rats have been variable but historically high, ranging from about 60% to nearly 100% (Bird et al. 1997, Dinsel et al. 2010). In contrast, Leydig cell adenomas range from 1% to 5% incidence in Sprague-Dawley rats, and in humans, testicular (and specifically Leydig cell) tumors constitute only 1% (and 0.03%, respectively) of all male human tumors (Foster 2007). The recent, extensive, and authoritative review of LCTs by Foster (2007) concluded

“Benign Leydig cell tumors (LCTs) (adenoma) are relatively common neoplastic findings found during routine animal carcinogenic bioassays … frequently present in older animals at termination (2 yr) and are not normally found after 1 yr of treatment. Normally, these tumors are not noted as a cause of death … rarely becoming malignant … there [being] a continuum between Leydig cell hyperplasia and adenoma. A large number of chemicals with widely differing structures have been shown to increase the appearance of LCTs when administered continuously in standard carcinogenicity bioassays … more frequently noted in rats. … Compound-induced LCTs are predominantly noted after exposure to agents, which do not directly interfere with DNA. In general, LCTs are rare tumors in human[s] (~3% of all testicular tumors) and when noted are normally in response to an underlying hormonal disturbance (e.g., gynecomastia, altered serum hormone levels). Malignant LCTs are also rare findings in humans (~10% of LCTs). Recent evidence has shown that Leydig cell hyperplastic nodules are relatively common findings in human testes at biopsy (and this would be from a highly selected population) for a variety of reproductive concerns. Morphological examination of the nodules would tend to support the notion that they are similar to the small LCTs commonly noted in rodents. … It certainly would be prudent to consider that agents that could produce these tumors in rodents should be considered as potential risks for human[s] and that they should not be dismissed based on their common occurrence in rodents versus their rarity as findings in human[s]. Thus far, no chemical has been shown unequivocally to produce LCTs in human[s].

… Nearly all hypothesized mechanisms for Leydig cell tumor production involve altered hormone levels that result in sustained elevation in luteinizing hormone (LH) that stimulates Leydig cell proliferation. Dopamine agonists [e.g., muselergine, bromocryptine, and cabergoline] decrease prolactin levels and thereby downregulate LH receptors on Leydig cells, resulting in reduced testosterone production and then a compensatory sustained increase in LH. A similar increase in LH is expected by agents that either block the androgen receptor (AR) (e.g., vinclozolin), inhibit testosterone metabolism (e.g., finasteride), or reduce testosterone during fetal development (e.g., butyl phthalate).”

These conclusions reflect a current scientific consensus that, while induced LCTs in bioassay experiments may indicate increased human tumor risk, LCTs generally never constitute a meaningful basis for linear cancer risk extrapolation assuming a genotoxic MOA, because these tumors arise in virtually all cases by nongenotoxic mechanisms that affect rates of testicular cell proliferation. In this regard, it is noteworthy that the high spontaneous incidence particularly of LCTs in the F344 rats was a key factor in recommendations that led to its replacement by the Sprague-Dawley strain of rats in the conduct of NTP bioassays for rat carcinogenicity (Kacew and Festig 1996, King-Herbert and Thayer 2006, Festig 2010).

Proposition 65 references and implements standard regulatory approaches to cancer bioassay interpretation. One common assumption applied in this context is that target-organ concordance is not a prerequisite for evaluating the implications of animal study results for humans (U.S. EPA 2005a). For example, elevated LCT risk contributed to regulatory determinations that vinclozolin exposures pose a potential human cancer risk (U.S. EPA 1996, CalEPA OEHHA 2006). The consensus conclusions from a workshop on the human relevance of rodent LCTs, sponsored by the American Industrial Health Council, the U.S. EPA, and the NIEHS, concluded that rats are an appropriate model for human Leydig cell growth control under some, but not all, conditions, but also that tumor-inducing MOAs known to affect Leydig cell growth rates, such as gonadotropin-releasing hormone agonists and dopamine agonists, are not relevant to humans (Clegg et al. 1997).

The overall weight of available evidence reviewed here, and in Section 4.2, supports the conclusion that elevated testicular
tumor incidence in male rats exposed to MTBE possibly by inhalation for 6 h/day, five days/week, for 18 months observed in the study reported by Chun et al. (1992) and Bird et al. (1997) were very likely caused by a nongenotoxic MOA mediated by dose-related hormone disruption, although a specific hormonal pathway for this has not yet been identified. Additionally, it may be that due to high CPN incidence in the two highest dose groups in this study, and the corresponding decreases in mean survival time, elevated rates of Leydig cell interstitial tumor incidence in the highest two dose groups were merely an artifact of hormonally mediated signals of cytotoxic stress experienced by those groups. As noted above for liver tumors, such an MOA would be associated with a nonlinear dose–response relationship.

5.3 Conclusions concerning tumorigenic MOAs for MTBE and TBA

The weight of available evidence reviewed here, supports the following conclusions:

1. Elevated rates of incidence of renal proximal tubule tumors in male F344 rats exposed chronically to MTBE by inhalation (Bird et al. 1997) and in male F344 rats exposed chronically to MTBE plus GVC by inhalation (Benson et al. 2011) were very likely caused by a nongenotoxic MOA mediated by male-rat-specific alpha2u-globulin accumulation and associated nephropathy, an MOA that is well recognized to be irrelevant to humans and the same MOA widely recognized to account for elevated rates of incidence of renal proximal tubule tumors in male F344 rats exposed to the MTBE metabolite TBA (see Section 5.2.1).

2. Thyroid follicular cell adenomas were significantly elevated in female B6C3F1 mice exposed to the highest drinking water concentration of TBA investigated, but because the only other tumor type known to be significantly elevated after chronic exposure to TBA is male rat renal tubule tumors mediated by an alpha2u-globulin MOA that is deemed irrelevant to humans (see point #1), the existing set of cancer bioassay data for TBA is inadequate to assess the potential human carcinogenicity of this MTBE metabolite. Furthermore, in the MTBE study by Burleigh-Fayer et al. (1992) conducted by inhalation in CD-1 mice, this finding was not reproduced (see Section 5.2.2).

3. Elevated liver tumor incidence in female CD-1 mice exposed chronically to MTBE by inhalation (Bird et al. 1997) were very likely caused by a nongenotoxic MOA mediated by dose-related hormone disruption (see Section 5.2.3).

4. Elevated testicular tumor incidence in male F344 and Sprague-Dawley rats exposed chronically to MTBE by inhalation (Chun et al. 1992, Bird et al. 1997) were very likely caused by a nongenotoxic MOA mediated by dose-related hormone disruption possibly related to selected cell killing in that tissue (see Section 5.2.4).

Conclusions 1 and 2 indicate that data sets available for rat kidney and mouse thyroid tumors are not relevant to characterizing the potential magnitude of MTBE cancer potency in humans. The two other tumor types listed above, mouse liver tumors and rat testicular tumors, provide at least some evidence of potential human carcinogenicity of MTBE. An assumption that there is no uncertainty in this regard corresponds to an estimated value of 1 for the likelihood \( P_{cancer} \) that appears in Approximation 1a (Section 1). Given the evidence reviewed in this section, assuming that \( P_{cancer} = 1 \) based on evidence for these two tumor types is reasonably conservative, but there is no scientific consensus concerning criteria that would allow a more accurate estimate of this likelihood given the relevant data currently available.

In each of the conclusions pertaining to mouse liver and rat testicular tumors, the phrase “very likely” is inherently imprecise, and its approximate, semi-quantitative definition requires subjective professional judgment informed by specific sets of relevant scientific information. The relevant information here is the combination of (A) evidence previously reviewed concerning whether MTBE exposure can be genotoxic to mammalian tumor-specific target stem cells in vivo with an LNT dose–response relationship, and (B) evidence previously reviewed concerning whether a purely genotoxic MOA in fact explains the increased incidence of each of the three types of tumor observed to be significantly elevated in experimental animals chronically exposed to MTBE.

Regarding evidence of type A, it was concluded that available genotoxicity data for MTBE and its metabolites indicates a relatively low (no greater than 10%) likelihood that MTBE exposure is both genotoxic to mammalian tumor-specific target stem cells in vivo and has an LNT dose–response relationship for induced genotoxicity.

Regarding evidence of type B, evidence indicates that a purely genotoxic MOA does not likely explain the increased incidence of each of the three types of tumor observed to be significantly elevated in experimental animals chronically exposed to MTBE. Instead, that evidence indicates that these observed increased tumor rates were more likely than not due entirely or nearly exclusively to one or more nongenotoxic MOAs that require cytotoxic precursor events that are either irrelevant to humans (as in the case of male rat alpha2u-globulin mediated nephrotoxicity induced by chronic exposure to MTBE or TBA), or that are not expected to have an LNT dose–response relationship at relatively low doses (as in the case of MTBE-induced alteration of hormone levels hypothesized to drive increased cell turnover, mineralization, and/or hyperplasia and associated tumorigenesis in mouse liver and rat testes).

Evidence of types A and B defined above jointly imply a chance no greater than approximately 10% that any of the tumor types 1–3 listed above were induced by chronic MTBE exposure through a genotoxic MOA. Assuming, conservatively, that uncertainty within this range is uniformly distributed, the expected value of this likelihood is estimated to be 5%. The complementary likelihood (100% \( \times P_0 \)) of a nongenotoxic MOA for MTBE-induced tumors is estimated to be 95%, which corresponds to the assumption that \( P_0 = 0.95 \) concerning the quantity \( P_0 \) that appears in Approximation 1a (Section 1).

5.4 Experiments to help identify an MOA for MTBE-induced tumors

Conclusions drawn in Section 5.3—that each case of significantly increased tumor incidence observed in rat kidney and
testes and mouse liver after chronic MTBE exposure involved an MOA unrelated to increased rate of mutation—can be investigated experimentally using transgenic mouse and rat assays that detect in vivo exposure-induced mutations in cells sampled from a variety of target tissues, including rat kidney and testes and mouse liver (e.g., Kanki et al. 2005, Wahnschaffe et al. 2005, Mei et al. 2006, Wang et al. 2010, Matsushita et al. 2013, OECD 2013). Although mutations are hypothesized to be critical, rate-limiting events required to cause tumor initiation and progression according to the multistage somatic mutation theory of cancer (Section 5.1), enhanced mutation frequencies are also clearly a characteristic feature of incipient and evolving tumor cells (Stephens et al. 2011). Therefore, to test whether MTBE is able to act effectively via a mutagenic MOA in vivo under rodent bioassay exposure conditions, in vivo mutation assays will need to be designed carefully to preclude or minimize detection of mutations that are contained in any (e.g., inadvertently) sampled tumor cells only as an indirect consequence, rather than as a cause, of MTBE-induced tumorigenesis. The usefulness of conducting such assays, however, depends on the relevance to humans of the tumor type being investigated. For example, in Section 5.2.1 we review evidence we conclude to be a sufficient basis for considering that observed MTBE-induced male rat kidney tumors are highly unlikely to have human relevance, although the experimental approach mentioned above could also be applied as an independent approach to confirm this conclusion.

6. Quantitative characterization of MTBE cancer potency

The cancer potency (CP) or “slope factor” (SF), for a chemical carcinogen is measured in units of inverse dose, (mg/kg/day)^{-1}, and represents the increased cancer risk per unit dose hypothesized to be caused by very low levels of exposure to that chemical, assuming an LNT dose–response relationship for increased risk in relation to dose (Anderson et al. 1983, U.S. EPA 1986, 2005a). This assumption is scientifically plausible, although still speculative, if a chemical carcinogen acts by a genotoxic MOA (see Section 5.1 and Supplementary Appendix C to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). If a chemical carcinogen does not act by a genotoxic MOA, but rather acts indirectly by inducing one or more cytotoxic precursor events that merely “promote” or accelerate the growth of spontaneous tumors, then it is scientifically more appropriate to estimate or bound cancer risk associated with chemical exposure by applying a traditional toxicological risk-management approach. By this approach, the no-observed-adverse-effect level (NOAEL), lowest-observed-adverse-effect level (LOAEL), or lower confidence limit (BMDL) on an estimated benchmark dose (BMD) for that precursor event endpoint is reduced by a magnitude referred to as an adjustment or uncertainty factor (AF), or a product of such factors, which serve to address applicable acute-to-chronic dose extrapolation, inter-species differences, human inter-individual differences, and any deficiencies in the available toxicity database, to derive a daily reference dose (RfD), or reference concentration in water (RfC_w), or the corresponding daily maximum allowed dose level for that chemical (U.S. EPA 2005a). Consequently, if a chemical carcinogen does not act by a genotoxic MOA, effectively zero estimable cancer risk is assumed to be associated with exposures below a calculated RfD or RfC_w for a toxic endpoint identified as being required for tumorigenesis (U.S. EPA 2005a).

Preferably, CP estimates are obtained from human epidemiological data; however, sets of such data that are adequate for risk assessment purposes are typically unavailable for environmental chemical carcinogens due to lack or limited sizes of defined exposed populations, and/or lack of ability or statistical power to account for potential confounding factors that complicate data interpretation (U.S. EPA 2005a). Since epidemiological data pertaining directly to MTBE-related cancer risk are not available, and so cannot form a basis for a direct estimate of carcinogenic potency in humans, cancer potency for MTBE was estimated from rodent bioassay data previously discussed that involve the two tumor types (rat testicular Leydig cell adenomas and CD-1 mouse liver tumors) determined to provide evidence that chronic MTBE exposure significantly elevates tumor incidence by a plausibly (but very unlikely, and as yet unverified) genotoxic MOA relevant to humans, which may or may not involve the MTBE metabolite formaldehyde. To perform these calculations, administered MTBE doses used in each bioassay considered were each first converted to the corresponding estimates of retained (i.e., non-exhaled) MTBE dose, using PBPK modeling results that were previously discussed, as indicated below. To calculate human-equivalent CP, it was assumed that at relatively low exposure levels of interest, humans would retain (and metabolize) 100% of MTBE absorbed by any route of exposure, and rodent-to-human pharmacodynamics adjustment factors were applied as described below. The assumption of 100% metabolism at low doses is conservative, because the efficiency of metabolism of any metabolized volatile compound is typically expected to be less than 100% even at very low doses, particularly in respiratory or dermal exposure scenarios (Bogen 1988, Bogen and Hall 1989). More refined estimates of the retained (metabolized) fraction of MTBE after human MTBE exposure by different routes, which could be obtained using a human PBPK model for MTBE (e.g., that of Rao and Ginsberg 1997, Licata et al. 2001, or Kim et al. 2007), are beyond the scope of this MTBE potency assessment.

To inform kidney-tumor-related MOA considerations discussed in Section 5, additional potency calculations were performed using three sets of bioassay data on renal tubule tumors in male rats chronically exposed to TBA in drinking water (NTP 1995, Cirvello et al. 1995), inhaled MTBE vapor (Bird et al. 1997), and inhaled MTBE plus GVC (Benson et al. 2011). In these calculations, TBA, as well as MTBE-related administered doses in each bioassay considered, were each first reexpressed as equivalent retained (i.e., non-exhaled) TBA dose, using PBPK modeling results, as described below. The potency estimate obtained using data involving TBA-exposed rats was then compared with those obtained using data involving rats exposed either to MTBE alone or to MTBE plus GVC.

Potency estimates are derived below for MTBE considering each of two possibilities concerning the MOA by which it may increase cancer risk after chronic exposure: either
6.1 Cancer potency assuming a nongenotoxic MOA

For reasons previously summarized, chronic MTBE exposure most likely has a nongenotoxic MOA for those tumor types that it has been shown to elevate. Applying a traditional toxicological risk-management approach in this case, the NOAEL or LOAEL for the most sensitive plausible precursor endpoint is reduced using a traditionally derived, combined AF (U.S. EPA 2005a). In the absence of known, specific precursor events critical to a nongenotoxic MOA for each elevated tumor type, a common surrogate precursor event applicable also to humans was conservatively assumed to be the most sensitive potentially adverse endpoint of any kind associated with chronic MTBE exposure in any species studied. As noted in Section 5.2.1, the most sensitive adverse endpoint identified among all those associated with chronic exposure to MTBE were significant kidney weight increases in male and female Wistar rats exposed to MTBE in drinking water for two years at all concentrations tested (Dodd et al. 2013). The lowest concentration tested was 0.5 mg/mL, corresponding to nominal average (± 1 SD) intakes of 25 ± 11 and 49 ± 14 mg/kg/day for male and female rats, respectively (Dodd et al. 2013). A PBPK-based relationship between administered drinking water concentration and retained MTBE dose was estimated for male rats based on PBPK-based estimated of retained dose reported by Borghoff et al. (2010) that correspond directly to the MTBE concentrations in water administered by Dodd et al. (2013) to male and female rats (see Figure 3, Section 2.2.1). The male rats in the Dodd et al. (2013) study exposed to the lowest concentration of MTBE in water (0.5 mg/mL) used in that study exhibited the lowest effective LOAEL, 25 mg/kg/day, expressed in units of mg/kg/day of MTBE estimated to be consumed over a 24-month exposure period, as calculated by Dodd et al. (2013) using data on mean BW, mean daily water consumption, and the analytical MTBE concentration. Borghoff et al. (2010) estimated that at concentrations of 0.5, 3.0, 7.5, and 15 mg/mL, male rats retain approximately 33, 23, 19, and 17% of consumed MTBE, respectively, and that female rats retain very similar percentages (see Table 2 of that study). The LOAEL concentration of 0.5 mg/mL for male rats in the Dodd et al. (2013) study therefore corresponds to a retained LOAEL of approximately 8.2 mg/kg/day.

To derive an RfD from the identified LOAEL, the LOAEL was divided by the product of the following AF values. The traditional AF value of 10 used for interspecies extrapolation was separated into PBPK and pharmacodynamics components, per EPA policy (U.S. EPA 1992, 2005a, pp. 3–6–3–7) discussed further below. In view of the PBPK-based approach to determine retained MTBE dose in rats and the assumption of 100% retention by humans, these PBPK and pharmacodynamic AF components were assumed to be 1 and 3, respectively, yielding a combined AF of 3 for interspecies extrapolation. Note that the AF value of 3 assumed here for interspecies pharmacodynamics differences is conservative, insofar as it is somewhat larger than the alternative value obtained explicitly by using the approximate average adult male rat BW of 0.60 kg indicated in Figure 1 of Dodd et al. (2013): \( AF = [(70 \text{ kg})/(0.60 \text{ kg})]^{1/8} = 1.8 \). A traditional AF value of 10 was used to address human inter-individual variability in sensitivity. Finally, an AF value of 1 was used to address LOAEL-to-NOAEL extrapolation, in view of BMD calculations that are discussed below in this subsection. Those calculations show quite clearly that the LOAEL in this case addresses a fairly subtle trend toward an adverse effect, rather than a frankly adverse effect using standard (U.S. EPA 2005a) methods of endpoint evaluation.

The combined AF applied was therefore \( 3 \times 10 \times 1 = 30 \), yielding an RfD value of \( (8.2 \text{ mg/kg/day})/30 = 0.27 \text{ mg/kg/day} \). Assuming water consumption of 2 L/day by a reference adult with a BW of 70 kg (U.S. EPA 2005a), this RfD corresponds to an RfCw of \( [(8.2 \text{ mg/kg/day})/30](70 \text{ kg})/(2 \text{ L/day}) = 9.6 \text{ mg/L} \) or 9,600 ppb (rounded to two significant digits). Since a traditional AF value of 10 was used to address human inter-individual variability in sensitivity, the calculated RfCw is designed to prevent the occurrence of any plausible carcinogenic precursor event in a general exposure population, including relatively sensitive individuals, assuming a nongenotoxic MOA. For an individual with a typical or median level of sensitivity exposed chronically to MTBE, or for an individual exposed for a small fraction of a lifetime, an appropriate RfCw would be 10-fold greater (96,000 ppb). Assuming a nongenotoxic MOA for any MTBE-associated increase in cancer risk, the cancer potency of MTBE is expected to be zero below the applicable RfCw.

As the point of departure for deriving an RfCw that protects against MTBE-related cancer risk under a nongenotoxic MOA assumption, an one-tailed 95% lower confidence bound (BMDL) on a corresponding BMD could be used instead of the LOAEL identified above (U.S. EPA 2005a). Using a two-tailed 95% confidence bound BMDL would be even more conservative. Two alternative benchmark response (BMR) values were considered: an increased relative weight equal to 110% of the predicted control-group relative weight, and an increase in relative weight equal to one control-group SD above the predicted control-group relative weight (U.S. EPA 2012c, at Section 2.2.2, p. 21–24). The first of these BMR definitions was determined to be the more sensitive (smallest) BMR for both the left and right kidney. Using this BMR definition, a BMD approach was applied to the continuous sets of data on increased relative weights of the left and right kidney listed in Table 5 of Dodd et al. (2013) for male rats tested in each MTBE concentration group, using unweighted least-squares linear regression implemented using Mathematica 8.0 (Wolfram Research 2012) to fit a linear dose–response model that was found to be consistent with both sets of data by F-test (\( p > 0.05 \)). The right kidney was determined to exhibit the lowest two-tailed BMDL (−31 mg/kg/day), which is greater than the LOAEL of 8.2 mg/kg/day, indicating that, for this endpoint, the LOAEL itself provides a more conservative indication of dose at which a potential adverse response could occur than the calculated BMDL. Consequently, the RfCw calculated using the traditional LOAEL-adjusted-by-AF approach applied above is more conservative (by a factor of 30.9/8.2 = −4) than a corresponding RfCw calculated using a BMD approach.
6.2 Cancer potency assuming a genotoxic MOA

CP estimation was done using LNT dose–response modeling methods very similar to those applied by EPA over the last 25 years (U.S. EPA 1986, 2005a), modified as explained below to address MOA uncertainty. Specifically, SF values were estimated by applying a GHS model algorithm (Bogen 2011) to each such set of bioassay data (see Supplementary Appendix D to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367 for modeling and calculation details). A complete cumulative probability distribution function (cdf) characterizing estimation error in SF, and a corresponding cdf for BMD were calculated using the GHS modeling approach. The cdfs obtained for SF and BMD allowed straightforward calculation of the corresponding one-tailed upper 95% and lower 5% confidence bounds (see Supplementary Appendix D). A complete cumulative probability distribution function (cdf) characterizing estimation error in SF, and the corresponding expected or arithmetic average values (<SF> and <BMD>), respectively (see Supplementary Appendix D).

For the purpose of potency estimation, the GHS model was applied to tumor-response data corresponding to the studies listed in Tables 3 and 4, together with the corresponding PBPK-based estimates of lifetime TWA dose noted in those tables. In cases where the duration of exposure (Tobs) differed from the period of observation (Tobs) measured in weeks, and/or Tobs differed from the standard rodent lifetime (Tlife = 104 weeks), the TWA dose (Dadj) for each bioassay was calculated from estimated retained dose D as Dadj = D S (T/Tobs) (Tobs/Tlife)3 (U.S. EPA 2005a). The latter definition of Dadj applies a rodent-interspecies PBPK-scaling factor of S = 1 in the case of rat bioassay data, and S = [(300 g)/(35 g)]0.25 in the case of mouse data (see Section 2.2.1, and footnotes g and h of Table 3 in Section 6.2). (The interspecies scaling factor S is further discussed in Supplementary Appendix D.2 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Resulting calculated estimates of expected potency (<SF>) and upper-bound potency (SF*) are listed in these tables for each data set. The estimated upper-bound rodent potency (SF*) values range ~100-fold from 0.00077 to 0.071 (mg/kg/day)−1. The greatest upper-bound potency estimate, more than 10-fold greater than the other three estimates, was obtained using data reported by Bird et al. (1997) for testicular tumors in male rats that were considered relatively unreliable by those study authors, as well as by CalEPA OEHHA (1999), as noted in Table 3 (footnote e).

Table 3. Cancer potencies estimated from rodent bioassay data sets indicating possible human cancer risk of chronic low-level MTBE exposure.a

| Study[a] [Duration] | Species, (BW) | Strain Route[b] | Sex | Tumor Type[b] | Rodent potency*[mg/kg/day]−1 | Human potency*[mg/kg/day]−1 |
|---------------------|---------------|-----------------|-----|---------------|------------------------------|------------------------------|
|                     |               |                 |     |               | <SF>                        | SF*                         |
| Belpoggi et al. (1995, 1998) [104/174] | Rat, SD (0.50 kg) | O*[a] | M | TLA | 0.00504 | 0.00150 |
|                     |               |                 |     |               | 0.00011 | 0.00028 |
| Bird et al. 1997; CalEPA OEHHA 1999*[a], p. 56, 81; [104/97–82] | Rat, F,344 (0.40 kg) | IN[t] | M | TLA | 0.0076e | 0.017e |
|                     |               |                 |     |               | 0.014e | 0.032e |
| Bird et al. 1997, CalEPA OEHHA 1999*[a], p. 58; [78/78] | Mouse, B6C3F1 (0.040 kg) | IN[g] | M | HC | 0.000011 | 0.00089 |
|                     |               |                 |     |               | 0.00028 | 0.0023 |
| Bird et al. 1997, CalEPA OEHHA 1999*[a], p. 58; [78/78] | Mouse, B6C3F1 (0.035 kg) | IN[f] | F | HAC | 0.0000072 | 0.000077 |
|                     |               |                 |     |               | 0.00019 | 0.0020 |

*aStudies included are those determined in Sections 3 and 4 to have provided evidence that chronic MTBE exposure significantly elevated tumor incidence by a plausibly genotoxic MOA relevant to humans. Duration = T/Tobs, where T = exposure duration in weeks (wk) and Tobs = duration(s) of observation (wk). For Bird et al. (1997) rat study, Tobs = 97 and 82 wk for mid and high dose groups, respectively.

*bBW = animal body weight (kg), O = oral gavage, IN = inhalation; TLA = Leydig interstitial cell testicular adenoma, HC = hepatocellular carcinoma, HAC = hepatocellular adenoma or carcinoma.

The frequency of these testicular adenomas in control animals (64%) from this study was lower than that normally reported, and below the laboratory’s previous control values of 86% and 91%. Thus, the increased incidences of testicular tumors in the 3,000 and 8,000 ppm [highest two exposure] groups reflect the lower incidence in the control group and may not be treatment related. Regarding this caveat, CalEPA OEHHA (1999, p. 81) concluded that “In view of this, the slightly divergent value for the potency estimate obtained with this data set is regarded with lower confidence than the other values obtained in this analysis,” and excluded the Bird et al. (1997) testicular tumor data from its result of statistical uncertainty … and biological uncertainties due to interspecies extrapolation and mode of action.”

*bExposure groups, administered [250, 1,000] mg MTBE/kg/day by gavage in oil, were estimated by Borgoff et al. (2010) to have retained [19, 12%], respectively (see Section 2.2.1), yielding corresponding estimated (mg/kg/day) values of retained dose D = (47.5, 120) and lifetime-adjusted TWA doses Dadj = D (104/174)(174/104)3 = (133, 336).

“SF” is one-tail upper 95% and lower 5% confidence bound on SF with respect to statistical parameter-estimation error. Listed values of <SF> and SF* were calculated using PBPK-based, lifetime time-weighted average estimates of retained MTBE dose for each study (see notes e–h). “Human potency” = human-equivalent potency = (Rodent potency) AF, where AF = interspecies pharmacodynamic adjustment factor = [(70 kg)/(BW)]0.25 (see Supplementary Appendix D and Section 3.10 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Resulting calculated estimates of expected potency (<SF>) and upper-bound potency (SF*) are listed in these tables for each data set. The estimated upper-bound rodent potency (SF*) values range ~100-fold from 0.00077 to 0.071 (mg/kg/day)−1. The greatest upper-bound potency estimate, more than 10-fold greater than the other three estimates, was obtained using data reported by Bird et al. (1997) for testicular tumors in male rats that were considered relatively unreliable by those study authors, as well as by CalEPA OEHHA (1999), as noted in Table 3 (footnote e).
Using the same approach, estimates of potency in units of inverse of dose measured as the retained amount of administered TBA, or instead as the retained amount of metabolized TBA, were also calculated for renal tubule tumors in male rats. These calculations allow the estimate based on data from the NTP (1995) rat bioassay involving administered TBA to be compared with two estimates based on data from two rat bioassays (Bird et al. 1997, Benson et al. 2011) in which an internal TBA dose was expected in the rats only as a metabolite of administered MTBE. In each case, as in Table 3, potency calculations were performed using PBPK-model-based estimates of retained TBA or retained TBA-equivalent dose. The resulting potency estimates are listed in Table 4, together with ratios of SF* estimated from data on rats administered MTBE to SF* estimated from data on rats administered TBA. Both ratios are greater than one, indicating that renal tubule tumors were produced less potently by TBA generated as a metabolite from MTBE than by TBA administered directly.

### 6.2.1 Animal-to-human extrapolation and age adjustment of tumorigenic potency

For tumor endpoints relevant to human risk extrapolation that are listed in Table 3, a human-equivalent potency was extrapolated from each listed estimate of potency for lifetime-exposed rodents using two alternative assumptions concerning interspecies “dose-scaling,” that is, concerning the method used to define a measure of dose (or “dose metric”) that is toxicologically equivalent for both rodents and humans. Rodent potency estimates were each calculated in units of reciprocal dose, (mg/kg/day)−1, using lifetime TWA dose measured in mg/kg/day. Assuming a 70-kg reference human adult, and bioassay rodents with an adult BW equal to W measured in kg, rodent-bioassay-based human-equivalent CP is traditionally estimated by EPA as the product of estimated SF and a dose-scaling factor (70 kg/W)1/8 or (70 kg/W)1/4 where the factor x is intended to reflect combined PBPK and pharmacodynamic interspecies differences (see Supplementary Appendix D.2 to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). Since PBPK considerations were addressed explicitly using PBPK models fit to appropriate data (see Section 2.2), conservative (i.e., upper-bound) or “best estimate” approaches used to extrapolate interspecies pharmacodynamic differences assumed that x = 1/8 or x = 0, respectively (see Supplementary Appendix D.2). Corresponding human-equivalent potency was thus estimated either as SF or as the product [(70 kg/W)1/8]SF*. Values of the latter, more conservative estimate of human-equivalent potency are listed in Table 3.

For CP estimated under a genotoxic MOA assumption for use with linear extrapolation of increased cancer risk, EPA currently assumes that there is a magnitude (Fearly) of increased relative CP during early life, where Fearly = 10 for the first 2 years, 3 for the next 14 years, and 1 for the remaining 56 years of a reference 70-year human lifespan (U.S. EPA 2005b). It would be appropriate to apply these factors, to the extent they are applicable to exposure scenarios of interest, to the potency estimates described below that are derived based on a genotoxic MOA assumption.

| Study[4][Duration] | Species (BW) Chemical | Strain Routeb | Sex | Tumor Typeb | Rat potencyc (mg retained TBA/kg/day)−1 | <SF*> | SF* | (SF* for TBA)/SF* |
|-------------------|----------------------|-------------|-----|-------------|-------------------------------------|------|-----|------------------|
| NTP (1995); Cirvello et al. (1995); [104/104] | Rat, F344/N (~0.45 kg) TBA | DWd | M | RTAC | 0.0018 | 0.0047 | 1 |
| Bird et al. 1997, CalEPA OEHHA 1999, p. 56; [104/97–82] | Rat, F344 (0.40 kg) MTBE | INb | M | RTAC | 0.00019 | 0.00092 | 5.1 |
| Benson et al. 2011, [104/104] | Rat, CDF(F344)CrlBR (0.40 kg) MTBE + GVC | INf | M | RTAC | 0.0015 | 0.0037 | 1.3 |

### Notes:

a Studies included are those determined in Sections 3, 4, and 5 to have provided evidence that chronic MTBE exposure significantly elevated tumor incidence by a plausibly genotoxic MOA relevant to humans. Duration is defined in note a of Table 3. For Bird et al. (1997) rat study, T20 = 97 and 82 wk for mid and high dose groups, respectively.

b BW = animal body weight (kg), DW = drinking water, IN = inhalation; RTAC = renal tubule adenoma or carcinoma; GVC = gasoline vapor condensate.

c SF = cancer potency or “slope factor” = increased risk per unit dose at low doses, <SF* > = expected value of SF. SF* = one-tail upper 95% confidence bound on SF with respect to statistical parameter-estimation error. Listed values of <SF*> and SF* were calculated using PBPK-based, lifetime time-weighted average estimates of retained TBA or TBA-equivalent dose for each study (see notes d–g).

d Exposed groups, administered [1,25, 2.5, 5.0] g/L TBA in drinking water for 2 years corresponding respectively to {85, 195, 420} mg/kg/day, were estimated in Section 2.2.1 (Figure 3) to have retained {60.68, 46.72, 29.80%}, yielding corresponding estimated retained, lifetime-adjusted TWA doses D = Dadj = [51.5, 91.1, 125] mg/kg/day.

e Of estimated retained (metabolized) dose D = [33, 148.8, 229] mg MTBE/kg/day (see Table 3 note f) corresponds to RMW D in units of mg TBA/kg/day, where RMW = 0.8408 = 74.12/88.15 = the ratio of TBA to MTBE molecular weights. Corresponding percentages of administered TBA retained by rats estimated in Section 2.2.2 (Figure 6) are {94.1, 78.5, 67.9%, respectively, yielding D = [22.9, 67.3, 85.9] and Dadj = D (104.97/82/104)2 = [22.9, 54.6, 42.1] in units of mg TBA/kg/day. Unordered Dadj and corresponding tumor-response data were re-sorted prior to potency analysis.

f Exposed groups, administered GVC combined with [0.40, 2.0, 4.0] mg/L or [110, 555, 1,110] ppm MTBE in air by inhalation 6 hr/day and 5 days/wk, were estimated by Benson et al. (2011) to have been administered MTBE doses of [165, 1240, 3272] mg/kg/day. The rat PBPK relationship between inhaled MTBE concentrations in air and retained TBA dose shown in Figure 2 (Section 2.2.1) was then applied to estimate corresponding retained MTBE doses of {9.57, 43.7, 76.2} mg/kg/day. Because a retained TBA dose of DMTBE, mg/kg/day is equivalent to an endogenously administered TBA dose of DMTBA = RMW DMTBE (see note e), calculated doses DMTBA of indirectly (metabolically) administered TBA were used to evaluate the relationship shown in Figure 6 (Section 2.2.2) to calculate corresponding estimates of retained TBA dose D = Dadj = [7.57, 28.6, 43.9] mg/kg/day that were used to estimate TBA potency from these bioassay data.
6.2.2 Upper-bound cancer potency assuming a genotoxic MOA

The upper-bound CP of MTBE assuming a genotoxic MOA is the maximum human-equivalent value of SF* calculated for relevant tumor endpoints based on relevant bioassay data sets. For this purpose, data involving male rat testicular tumors reported in the study by Bird et al. (1997) were excluded from consideration, because these data are considered relatively unreliable. According to Bird et al. (1997), “The frequency of these testicular adenomas in control animals (64%) from this study was lower than that normally reported, and below the laboratory’s previous control values of 86% and 91%.

Thus, the increased incidences of testicular tumors in the 3000 and 8000 ppm [highest two exposure] groups reflect the lower incidence in the control group and may not be treatment related.” Regarding this caveat, CalEPA OEHHA (1999, p. 81) concluded “In view of this, the slightly divergent value for the potency estimate obtained with this data set is regarded with lower confidence than the other values obtained in this analysis.” For this reason, CalEPA OEHHA (1999, p. 81) excluded the Bird et al. (1997) testicular tumor data from those it considered to calculate a “preferred” quantitative estimate of MTBE cancer risk, noting that “it is plausible that the lower bound on the human [value of SF] includes zero” as “a result of statistical uncertainty … and biological uncertainties due to interspecies extrapolation and mode of action.” Of the remaining three estimated values of SF*, the maximum value is SF* = 0.0028 (mg/kg/day)^–1. This value represents a conservatively defined plausible upper bound on the CP (Q) of MTBE for exposed humans. SF* here also represents the value of the quantity Q_{MOA} that appears in Approximation 1b (Section 1). This corresponds to a conservative (i.e., upper-bound) assumption that there is zero likelihood that MTBE-induced tumors have a nonlinear, nongenotoxic MOA, which is equivalent to assuming that P_0 = 0 in Approximation 1a, such that Q_{MOA} = Q = SF*.

Another approach that can be applied to estimate a plausible upper bound on CP is to define this value as the geometric mean of SF* estimates that do not differ from each other by more than a factor of two or three. For example, CalEPA OEHHA (1999) used this approach to derive its upper-bound estimate of MTBE CP. Since the other two estimates of human-equivalent SF* listed in Table 3 are only slightly less than 0.0028 (mg/kg/day)^–1, applying the geometric mean approach yields an upper-bound CP estimate of 0.0023 (mg/kg/day)^–1, a value only slightly (~18%) lower than the maximum relevant SF* value of 0.0028 (mg/kg/day)^–1 listed in Table 3.

6.2.3 Expected cancer potency assuming a genotoxic MOA

Expected values of CP, denoted <SF>, that are listed in Table 3 each represent the arithmetic mean value of CP with respect only to one specific source of uncertainty: statistical error associated with SF-parameter estimation. Assuming that LNT extrapolation of increased cancer risk is justified by a genotoxic MOA for a particular carcinogen, only the expected value of the potency of that carcinogen can be multiplied by average dose (d) to yield a corresponding expected value (<R>) of the population-average value of individual increased risk, and only this specific estimate of risk can be multiplied by the exposed population size (N) to calculate the corresponding expected number (n) of additional cancer cases predicted to be due to exposure of that population to that carcinogen, specifically as n = <R> N = <SF> d N (Bogen and Spear 1987, NRC 1994).

To calculate an expected value of CP, calculated potency must be averaged arithmetically with respect to all relevant sources of uncertainty, including (1) parameter estimation error, (2) interspecies extrapolation uncertainty, (3) uncertainty regarding which data set is most predictive of human cancer risk, and (4) “model” uncertainty addressed by an estimated likelihood that a genotoxic MOA for MTBE is true. The estimates <SF> in Table 3 all address uncertainty source #1. Uncertainty source #2 is addressed by the distinction between <SF> estimates listed in Table 3 under the heading “Rodent potency” versus those listed under the heading “Human potency.” As noted above (Section 6.2), rodent potency estimates are human-equivalent potencies under the interspecies extrapolation assumption that the interspecies extrapolation exponent (x) is equal to zero. Potency estimates listed under the “Human potency” heading incorporate the more conservative alternative assumption that x = 1/8, which is applicable in view of the PBPK-based estimates of retained MTBE dose that were used to calculate all potency estimates listed in Table 3. Uncertainty source #3 concerns the fact that, besides the <SF> estimates based on data for male rat testicular tumors reported by Bird et al. (1997) and excluded for reasons discussed previously and in footnote e of Table 3, a total of six estimates of <SF> appear in Table 3 that were calculated using different bioassay data sets, comprising a set of three estimates under the “Rodent potency” heading corresponding to an interspecies extrapolation assumption that x = 0, and a set of three estimates under the “Human potency” heading corresponding to an interspecies extrapolation assumption that x = 1/8. Finally, uncertainty source #4 was characterized above (Section 5.3) as an estimated likelihood of 5% that a genotoxic MOA for MTBE is true, implying a 95% chance that MTBE CP at very low doses is effectively zero (corresponding to the nongenotoxic MOA assumption).

The expected value of potency with respect to the first three sources of uncertainty discussed in the previous paragraph is just the arithmetic mean of potencies calculated from each of the data sets, and each of the methods for interspecies potency extrapolation, that are plausibly equally predictive for the purpose of estimating the expected value of human CP (Bogen 1995b). Therefore, the expected value of potency with respect to all four sources of uncertainty discussed in the previous paragraph is just the likelihood that a genotoxic MOA for MTBE is true, multiplied by the arithmetic mean of the six non-excluded values of <SF> listed in Table 3:

\[
<SF> = 0.05 \times (\text{arithmetic mean of the 6 } <SF> \text{ values listed in Table 3}) \\
= 0.05 \times 0.00036 \text{ (mg/kg/day)}^{-1}
\]

\[
= 0.00018 \text{ (mg/kg/day)}^{-1}
\]

A potency value of \( <SF> = 1.8 \times 10^{-5} \text{ (mg/kg/day)}^{-1} \) thus represents a reasonable expectation of the magnitude of increased cancer risk to humans exposed to the possible human chemical carcinogen MTBE. The value <SF> also represents the value of the quantity Q_{MOA} that appears in Approximation 1b (Section 1).
6.3 Summary of estimated MTBE cancer potency

The foregoing CP assessment for MTBE supports conclusions summarized in Table 5 concerning a plausible upper bound, a reasonable expectation, and a most likely value for the CP (Q_{MOA}) of MTBE to exposed humans, assessed in a way that addresses MOA considerations. The corresponding cancer risks may be calculated using Approximation 1b discussed in Section 1. Table 5 also lists the corresponding annual risks per ppb MTBE in water consumed by a reference adult assuming MTBE has a genotoxic MOA, and the RfC_w, calculated in Section 6.1 assuming MTBE has a nongenotoxic MOA, for increased cancer risk.

7. Discussion

The weight of available evidence reviewed indicates that elevated rates of incidence of renal proximal tubule tumors in male F344 rats exposed chronically to MTBE by inhalation were very likely caused by a nongenotoxic MOA mediated by male-rat-specific alpha2u-globulin accumulation and associated nephropathy—a pathway well recognized to be irrelevant to humans, which also is widely recognized to explain increased renal proximal tubule tumors observed in male F344 rats exposed to the MTBE metabolite TBA. The comparable or greater relative value of estimated potency of directly administered TBA, compared with potencies for TBA generated from MTBE, that are listed in Table 4 (Section 6.2) is consistent with the hypothesis that TBA generated by MTBE metabolism results in peak TBA concentrations in kidney that are comparable to or less than those generated by administration of TBA itself. Although this hypothesis could be assessed quantitatively using PBPK model predictions, basic PBPK considerations imply that a peak concentration of a compound generated only as a relatively slowly formed metabolite will be less than (i.e., damped out) relative to the peak concentration achieved if that compound is instead administered directly at the same dose. Our review of bioassay data indicating that MTBE and TBA can induce male rat kidney tumors (Sections 4.1 and 4.3) noted a convergence of published assessments that TBA has an alpha2u-globulin-nephropathy MOA for inducing kidney tumors (Section 5.2.1). TBA is an MTBE metabolite that is systemically circulated with obvious access to the kidney, and both compounds increase tubule cell proliferation and cause kidney tumors. MTBE induces renal tubule hyaline droplets in male but not female rats exposed subchronically to MTBE, increases kidney weights, and induces renal tubule cell proliferation, consistent with a male-rat-specific alpha2u-globulin nephropathy MOA for kidney-tumor induction. Consequently, we excluded MTBE-induced kidney tumors from among the other tumor endpoints (with greater average potency) that we used for the purpose of quantifying MTBE carcinogenic potency. However, it is noteworthy that including potency estimates from positive kidney tumor endpoints that we excluded would only reduce our expected and upper-bound MTBE potencies, with little effect on the latter. In this respect, we took a conservative approach to relevant MOA data to characterize current uncertainty in the human CP of MTBE quantitatively.

Other tumor types potentially biologically relevant to humans that were observed to be clearly elevated by chronic respiratory MTBE exposure are testicular tumors in male F344 and Sprague-Dawley rats and liver tumors in male and female CD-1 mice (Section 4.1). The effects of MTBE on mouse liver tumors and rat testicular tumors provide at least some evidence of potential human carcinogenicity of MTBE. An assumption that there is no uncertainty in this regard corresponds to an estimated value of 1 for the likelihood P_{cancer} that appears in Approximation 1a (Section 1). It is reasonably conservative to assume that P_{cancer} = 1, based on evidence for these two tumor types, but there is no scientific consensus concerning criteria that would allow a more accurate estimate of this likelihood given the relevant data currently available.

After reviewing related available MOA-related information (Sections 3.2, 4.2, 4.4, 5.2.3, and 5.2.4), we consider it very unlikely (yet not scientifically implausible) that MTBE-generated formaldehyde genotoxicity caused these observed elevated tumor rates, and very likely that these elevated rates were instead due to one or more nongenotoxic MOAs mediated by dose-related hormone disruption and associated mitogenic effects in the affected tissues. We reviewed the overall weight of scientific evidence concerning the likelihood (1-P_0) that these exposure-related tumors were caused by a genotoxic MOA consistent with an LNT model of increased tumor risk (Sections 5.3). Based on that review, we evaluated this likelihood to be 5% (i.e., we assumed that P_0 = 0.95) for the purpose of evaluating Approximation 1a (Section 1) for potency characterization (Section 6.2.3).

Estimates of CP (Q_{MOA}) of MTBE presented in Section 6, summarized in Table 5, were calculated in ways that address MOA-related uncertainty from three different perspectives. Human cancer risks may be calculated from these potency estimates using Approximation 1b discussed in Section 1.

| Assumed MOA         | Human Risk Measure | Unit         | Type of Estimate | Symbol | Value    |
|---------------------|-------------------|--------------|-----------------|--------|----------|
| Genotoxic Potency   | (mg/kg/day)^-1    | Average      |                 |  0.000018 |
| Genotoxic Annual Risk Per ppb | (ppb year)^-1 | Average      |                 |  7.3 x 10^-12 |
| Genotoxic Potency   | (mg/(ppb year))^1 | Upper bound  | SF*             |  0.0028  |
| Genotoxic Annual Risk Per ppb | (ppb year)^-1 | Upper bound  |                 |  1.1 x 10^-9  |
| Nongenotoxic Reference Water Concentration | ppb | Lower bound | RfC_w | 9,600    |

*Potency = lifetime increased cancer risk per unit time-weighted average dose at relatively low doses, assuming a chronic scenario of MTBE exposure to a reference 70-kg adult who has an average level of susceptibility relative to others. Risk would be n-fold greater for a person who has n-fold greater susceptibility relative to those of average susceptibility. Annual risk per ppb = Potency × (2 L/day) × (0.001 mg/L/ppb) × (1 year)/([70 kg] × [70 years]).

Under a nongenotoxic MOA assumption, exposure to water containing MTBE at a concentration below RfC_w is considered very unlikely to increase cancer risk, even in relatively susceptible individuals.
Human cancer risks that can be calculated using each of the different types of potency estimates presented are appropriate for different corresponding decision contexts (see, e.g., Bogen and Spear 1987, NRC 1994, Bogen 1995b; Bogen et al. 2009, and Supplemental/Online Materials. Supplementary Appendix C to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367). For example, human cancer risk calculated using the upper-bound estimate (SF\*) for MTBE potency (Section 6.2.2) would traditionally be applied in a public health regulatory (or regulatory-compliance) decision-making context. Figure 12 shows that the SF\* estimate obtained for MTBE falls below more than 90% of the 81 SF\* values recently listed by the U.S. EPA (2013) IRIS.

Section 5.4 discussed additional forward mutation assays using transgenic mice and rats that could be applied to determine whether MTBE exposure is capable of inducing mutations in vivo in those specific target tissues (mouse liver, and rat kidney and testes) in which chronic MTBE exposure has been observed to elevate tumor incidence rates. Such experiments are currently feasible and would likely contribute fundamental insight concerning the tumorigenic MOA of MTBE. If increased mutation rates fail to occur in rodent target tissues after a tumorigenic MTBE exposure regime, but positive-control experiments show that sustained exposure to mutagenic carcinogens in each case acts to increase mutation rates in the same tissues in which MTBE elicits tumors, then a mutation-driven MOA for MTBE-induced tumors would not be plausible. Such a result would add to existing evidence indicating that application of a traditional safety factor or BMD approach, rather than LNT risk extrapolation, is scientifically the most appropriate approach to characterize potential human cancer risk associated with low-level environmental exposures to MTBE. In contrast, detection of increased mutation rates in tumor-target tissues by forward mutation assays using transgenic MTBE-exposed mice and rats would provide clear evidence that a genotoxic MOA for MTBE is scientifically plausible, and support the application of LNT extrapolation of MTBE cancer risks in the context of health-protective regulatory decision making. In the latter case, for reasons discussed in Section 5.1, there would still be substantial uncertainty concerning the true magnitude of increased cancer risk that may be caused by very low-level human exposures to MTBE. Thus ultimately, experimental data and improved theory concerning fundamental molecular mechanisms of tumorigenesis continue to be the long-recognized key to improved risk assessment for chemical carcinogens such as MTBE (Albert 1994).

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Declaration of interest

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Supplementary material available online

Supplementary Appendices A–E to be found online at http://dx.doi.org/10.3109/10408444.2015.1052367