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Investigation of the potential mutagenicity of ethyl tertiary-butyl ether in the tumor target tissue using transgenic Big Blue Fischer 344 rats following whole body inhalation exposure

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Abstract

Ethyl tertiary-butyl ether (ETBE) is a fuel oxygenate used for the efficiency of motor vehicle fuels and their octane ratings. ETBE has been reported to induce liver adenomas in male rats in a 2-year bioassay at the highest inhalation concentration tested of 5000 ppm. To investigate the potential mutagenicity of ETBE in the liver, male Big Blue Fischer 344 rats were exposed for 28 consecutive days (6 h/day) to 0, 500, 1500, and 5000 ppm ETBE. The treated rats were sacrificed 3 days post-exposure and the frequencies of *cII* mutants were evaluated in the liver and bone marrow tissues. The mutant frequency (MF) of the liver in the negative control group was 36.3×10^{-6} and this value was not significantly different in ETBE-exposed animals (39.4, 37.3, and 45.9×10^{-6} in 500, 1500, and 5000 ppm groups, respectively). In the bone marrow, the mean MF in the negative control was 32.9×10^{-6} which was not different from the means of the exposed groups (33.8, 22.6, and 32.0×10^{-6} for groups exposed to 500, 1500 and 5000 ppm, respectively). These data, along with consistent negative response reported in the literature for other apical genotoxicity endpoints informs that mutagenicity is not likely the initial key event in the mode of action for ETBE-induced hepatocarcinogenesis in the rat.

KEYWORDS
bone marrow, *cII* mutation, ethyl tertiary-butyl ether, liver, mode of action, tumor

1 | INTRODUCTION

Ethyl tertiary-butyl ether (ETBE; CAS: 637–92-3; synonym: 2-ethoxy-2-methy propane) is produced globally from natural gas derived isobutylene and ethanol from biomass. Its primary use is as an oxygenated fuel to enhance the efficiency of motor vehicle combustion which reduces air pollution and CO_2 from transportation. ETBE is approved for use in motor vehicles in the United States and is used to meet CO_2 reduction targets in Japan, Argentina, and several European countries. Inhalation of gasoline vapors is the primary potential route of human exposure to ETBE.

ETBE has been evaluated for carcinogenicity in Fischer 344 rats following inhalation exposure for 104 weeks (6 h/day, 5 days/week) at concentrations of 0, 500, 1500, and 5000 ppm (Saito et al., 2013). The only tumor finding in this study was a significant increase in hepatocellular adenomas in male, but not female, rats exposed to

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5000 ppm (18% vs. 0% in controls). No increases in liver tumors were observed in Sprague Dawley rats following oral gavage dosing of ETBE in corn oil (up to 1000 mg/kg/day; 4 days/week) for 104 weeks (Maltoni et al., 1999) or by administering it in drinking water to Fischer 344 rats up to a dose level of approximately 550 mg/kg/day for 104 weeks (Suzuki et al., 2012).

Currently, the mode of action (MoA) responsible for the increased liver tumor incidence in ETBE-exposed rats is not fully elucidated. There is limited evidence suggesting that nuclear receptor activation/binding (CAR, PXR and PPARα) might play a role in the hepatocarcinogenesis seen with ETBE (Kakehashi et al., 2013); however, these data are not conclusive. ETBE has been shown to be uniformly negative in the standard battery of genetic toxicology assays that included the bacterial reverse mutation test, an in vitro mammalian cell gene mutation assay (CHO/*Hprt*), a cytogenetic assay using CHO cells in culture and several in vivo erythrocyte micronucleus tests (see reviews by McGregor, 2007; USEPA, 2021). In contrast, Weng et al. (2011, 2012, 2013, 2014, and 2019) reported increases in oxidative DNA damage and strand breaks in various tissues, including the liver, of mice exposed by inhalation to ETBE. These authors also reported that ETBE-induced DNA damage is enhanced in *Aldh2* deficient mice compared to their wild-types.

The purpose of the study reported here was to investigate whether inhalation exposure to ETBE can induce mutagenicity in the tissue that has been identified as the tumor target. Towards this end, induction of *cII* mutations was evaluated in the livers of transgenic Big Blue rats. The results from this study are hoped to contribute to further the knowledge on the potential MoA responsible for the tumorigenicity of inhaled ETBE.

2 | MATERIALS AND METHODS

2.1 | Test substance

ETBE (Lot # EFOA0319; 96.13% pure) was provided by LyondellBasell (Channelview TX, USA). The test material was kept in a room set to maintain 18 °C to 24 °C under inert gas (nitrogen). The vehicle for the study was filtered air.

2.2 | Test animals

The study was approved by the conducting laboratory's Institution Animal Care and Use Committee. Fischer 344 Big Blue® homozygous male transgenic rats were received from Taconic Biosciences (Germantown, NY) at 8 weeks old with weights between 215 and 257 grams. Upon receipt, each animal was identified using a subcutaneously implanted electronic identification chip (BMDS system). Animal rooms were maintained with target temperatures of 68 °F to 78 °F, relative target humidity of 30% to 70% and a 12- hour light/12-hour dark cycle. Ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms. PMI Nutrition International, LLC Certified Rodent LabDiet 5002 was provided ad libitum except during inhalation exposures. Municipal water treated via reverse osmosis and ultraviolet irradiation was freely available via an automatic watering system except during inhalation exposures.

2.3 | Study design

The study was performed as per the OECD Test Guideline 488 (2013) with the specific objective of evaluating mutagenicity in the tumor target tissue. Animals were assigned to various groups as shown below by a stratified randomization scheme designed to achieve similar group mean body weights.

Group no.	Treatment	Target exposure concentration (ppm)	Daily exposure duration (hours)
1	Filtered air	0	6
2	ETBE	500	6
3	ETBE	1500	6
4	ETBE	5000	6

Given the purpose of the study, the highest exposure concentration was anchored to the dose that induced hepatocarcinogenesis. In the cancer bioassay, a significant increase in the incidence of hepatocellular adenomas was noted in male rats exposed to 5000 ppm ETBE. Furthermore, the 5000-ppm exposure level also translated to the limit concentration of 20 mg/L as recommended in the OECD Test Guideline 412 for 28-day repeat-dose inhalation studies for vapors.

2.4 | Exposure methods

Humidified supply air and dry compressed air were mixed and delivered to the chamber with compressed nitrogen to provide a comparable relative oxygen content in all exposure chambers. For each chamber, ETBE was delivered to a single J-type glass-bead column vaporization system. Vaporization occurred as the test substance flowed over the surface of heated beads, while nitrogen flowed up through the column. The concentrated vapors were directed through polyethylene tubing to a stainless-steel manifold, where they were subsequently directed to a flowmeter controlled with a needle valve designated for each exposure group. Vapors from the flowmeters were directed to the chamber inlet where the vapors mixed with humidified supply air and dry compressed air. ETBE concentration within the exposure chambers was sampled and analyzed approximately every 30 to 45 minutes using a gas chromatograph (GC). Samples were collected from the approximate animal-breathing zone of each exposure chamber via Teflon® tubing. An In-quadratic equation based on the GC calibration curve was used to calculate the measured concentration in ppm. Animals were exposed for 28 consecutive days, 6 h/day.

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TABLE 1 *cII* mutant frequency in livers of big blue rats exposed by inhalation to ETBE

Group	Animal no.	No. of packagings	No. of phage screened	No. of mutants	*cII* MF ($\times 10^{-6}$)	Mean MF (±SD)
0 ppm	1001	2	233,691	7	30.0	
	1002	2	336,277	13	38.7	
	1003	2	279,663	11	39.3	
	1004	2	202,617	7	34.5	
	1005	3	383,951	15	39.1	36.3 ± 4.1
500 ppm	2001	2	248,589	8	32.2	
	2002	3	307,502	17	55.3	
	2003	3	359,263	15	41.8	
	2004	2	272,001	6	22.1	
	2005	2	219,303	10	45.6	39.4 ± 12.7
1500 ppm	3001	3	605,298	16	26.4	
	3002	2	253,272	10	39.5	
	3003	2	244,333	9	36.8	
	3004	2	302,223	12	39.7	
	3005	3	203,483	9	44.2	37.3 ± 6.6
5000 ppm	4001	2	260,082	14	53.8	
	4002	3	382,674	17	44.4	
	4003	2	249,866	15	60.0	
	4004	2	391,613	6	15.3	
	4005	2	285,197	16	56.1	45.9 ± 18.1
Positive control	437	3	316,270	78	246.6	
	438	2	279,663	60	214.5	
	439	3	293,284	64	218.2	
	440	2	284,430	40	140.6	
	441	2	301,372	68	225.6	209.1 ± 40.3[a]

[a]Statistically significant (1-Way ANOVA, $p < .001$), compared to 0 ppm.

2.5 | Observations

Animals were observed for the standard endpoints, including mortality/viability, detailed clinical observations, and cage side observations. Animals were weighed individually on Day 1 (prior to exposure), weekly (±1 day) during the study and at the time of scheduled necropsy. Food consumption was quantitatively measured weekly (±2 days) starting on Day 1 and continuing throughout the study.

2.5.1 | Necropsy

Animals were necropsied on day 31 (i.e., 3 days after the final exposure). Bone marrow was collected from both femurs and suspended using cold phosphate-buffered saline (PBS). Samples were aliquoted and held on wet ice during processing. The bone marrow was centrifuged, the supernatant decanted, and the resulting pellets flash frozen in liquid nitrogen. The entire liver was weighed but only the median lobe was frozen for use in the mutation assay. Liver and bone marrow samples were flash frozen in liquid nitrogen and stored frozen at approximately −70°C until the extraction of DNA.

2.5.2 | Extraction and packaging of genomic DNA

The tissues analyzed for mutagenicity were the liver and the bone marrow. Liver was the primary focus of the study while the bone marrow was selected to represent a rapidly proliferating tissue to ascertain the influence, if any, of the proliferative status of the tissue on the recovery of mutants. Frozen tissues of the first five animals in each group were processed using the RecoverEase DNA isolation kit from Agilent Technologies (Santa Clara, CA). DNA from frozen tissues of a previous study where five Big Blue® rats were treated by oral gavage with 20 mg/kg/day of ethyl nitrosourea (ENU) on Days 1, 2, 3, 10, 17 and 24 with necropsy on Day 31 was used as a concurrent packaging control for the positive control group. Isolated DNA was processed using Packaging Reaction Mix (PRM; New York University, New York, NY) and Agilent instruction manuals for transgenic shuttle vector recovery. Frozen stocks of E. coli strain G1250 (Agilent) were used to prepare master bacterial plates and overnight suspension. The

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TABLE 2 *cII* mutant frequency in bone marrow of big blue rats exposed by inhalation to ETBE

Group	Animal No.	No. of packagings	No. of phage screened	No. of mutants	*cII* MF ($\times 10^{-6}$)	Mean MF (±SD)
0 ppm	1001	2	337,554	16	47.4	
	1002	2	229,009	3	13.1	
	1003	2	252,846	6	23.7	
	1004	2	205,171	5	24.4	
	1005	2	357,134	20	56.0	32.9 ± 18.0
500 ppm	2001	3	276,683	10	36.1	
	2002	2	287,325	12	41.8	
	2003	3	292,859	6	20.5	
	2004	2	372,033	15	40.3	
	2005	2	296,690	9	30.3	33.8 ± 8.7
1500 ppm	3001	2	298,818	8	26.8	
	3002	2	297,115	2	6.7	
	3003	3	390,762	10	25.6	
	3004	2	251,995	7	27.8	
	3005	3	455,463	12	26.3	22.6 ± 8.9
5000 ppm	4001	2	218,963	5	22.8	
	4002	2	349,047	9	25.8	
	4003	2	232,840	9	38.7	
	4004	2	242,204	10	41.3	
	4005	2	320,101	10	31.2	32.0 ± 8.0
Positive control	437	3	384,803	175	454.8	
	438	2	379,695	128	337.1	
	439	4	383,100	153	399.4	
	440	2	395,019	186	470.9	
	441	2	377,141	184	487.9	430.0 ± 61.6[a]

[a]Statistically significant (one-way ANOVA, $p < .001$), compared to 0 ppm.

packaged phage was adsorbed onto E. coli G1250 suspension cultures for at least 30 minutes, molten top agar added, and the cells were plated onto bottom agar plates. The plates were incubated overnight at 37 ± 1.0°C, then scored for plaque formation and titer determination; *cII* mutant selection plates were incubated for 2 days at 24 ± 0.5°C and scored for mutant plaques. At least 125,000 phage were evaluated from at least two packagings for mutant frequency determination.

2.5.3 | Data evaluation

Body weight, food consumption and organ weight data were analyzed by non-parametric Kruskal-Wallis test followed by Dunn's test for pair-wise comparisons. Log10 transformed mutant frequency data were compared by one-way ANOVA followed by Dunnett's test for pair-wise comparisons if the data were normally distributed with equal variance or by non-parametric Kruskal-Wallis test followed by Mann–Whitney test for pair-wise comparisons. The test substance was considered positive if it induced a statistically significant increase in the frequency of *cII* mutants at any dose level, and the frequency in the treated group was outside the 95% control limits (CL) of the historical background mutant frequency range. Biological significance and dose–response were an important consideration in the final determination of a positive response.

TABLE 3 Laboratory historical (2014–2018) Big Blue rat negative control *cII* mutant frequency ($\times 10^{-6}$)

	Liver		Bone marrow	
	Individual animals	Studies	Individual animals	Studies
Mean	237.2	233.5	30.5	30.5
SD	128.8	107.6	14.0	5.7
95% control limits	0.0–494.8	18.3–448.7	2.5–58.5	19.1–41.9
Range	60.8–614.1	139.5–389.9	12.3–71.7	19.3–38.0

3 | RESULTS

Mean analyzed exposure concentrations in the chambers were 0, 507 (±20.4), 1524 (±56.1), and 5054 (±231.5) ppm for Groups 1–4, respectively. All animals survived to the scheduled necropsy. There were no

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TABLE 4 Laboratory historical (2014–2018) big blue rat positive control *cII* mutant frequency ($\times 10^{-6}$)

	Liver		Bone marrow	
	Individual animals	Studies	Individual animals	Studies
Mean	237.2	233.5	396.2	396.2
SD	128.8	107.6	204.9	182.5
95% control limits	0.0–494.8	18.3–448.7	0.0–806.0	31.2–761.2
Range	60.8–614.1	139.5–389.9	166.0–1030.6	201.5–650.3

Note: Pooled data: 20 mg/kg/day ENU dosed on Days 1, 2 and 3 and necropsied on Day 31; dosed on Days 1, 2, 3, 10, 17 and 24 and necropsied on Day 31; dosed on Days 1, 2, 3, 12, 19 and 26 and necropsied on Day 31.

clear or consistent test substance related clinical observations. Body weights were unaffected by exposure to ETBE. Statistically significant lower mean body weight gain was noted in the 5000-ppm group from Days 1 to 8 (14.7 g in 0 ppm vs. 6.7 g in 5000 ppm groups). Mean body weights remained comparable to the control group throughout the study. The lower mean body weight gain in the 5000-ppm group also correlated with decreased food consumption during the same interval (17.57 g in control vs. 15.14 g in 5000 ppm). Statistically significant lower food consumption was also noted in the 1500 ppm (16.38 g) and 5000 ppm (16.10 g) groups compared to 0 ppm group (17.69 g) from Days 8 to 15. Food consumption values for all groups were comparable to the concurrent control during the final 2 weeks of exposure. Mean absolute (13.18 g) and relative (to body weight) liver weights (4.36 g) of the 5000-ppm group were significantly higher than the respective control values of 11.28 and 3.73 g.

The mutant frequency (MF) of the liver in the negative control group was 36.3×10^{-6} (Table 1) and this value was comparable to the historical mean MF of 48.3×10^{-6} for this tissue (Table 3). MF of individual control animals were within the 95% CL of the historical background MF. Mean MF in livers from ETBE-exposed animals (39.4, 37.3, and $45.9 \pm 18.1 \times 10^{-6}$ in 500, 1500, and 5000 ppm, respectively, Table 1) were not significantly different from the control value. MF of individual ETBE-exposed animals were within the 95% CL of the historical background MF. Results from positive control treated animals demonstrated significantly elevated MF of 209.1×10^{-6}, which was comparable to the historical experience of 237.2×10^{-6} for this tissue (Table 4).

In the bone marrow, the mean MF in the negative control was 32.9×10^{-6} (Table 2) which is comparable to the historical MF of the performing laboratory (30.5×10^{-6}; Table 4). MF of individual negative control animals were within the 95% CL of the historical data. Exposure to ETBE did not significantly affect the mean MF (33.8×10^{-6}, 22.6×10^{-6}, and 32.0×10^{-6} in groups exposed to 500, 1500 and 5000 ppm, respectively). MF of individual ETBE-exposed animals were within the 95% CL of the historical background MF (Table 3). MF in ENU-treated animals were statistically elevated over the concurrently analyzed negative controls for this tissue (Table 4).

4 | DISCUSSION

Significant increase in the incidence of hepatocellular adenomas was observed in male F344 rats at the highest exposure concentration of 5000 ppm ETBE in a chronic inhalation bioassay (Saito et al., 2013). Toxicokinetic studies have demonstrated that ETBE is systemically available following inhalation exposure of rats, and its metabolism is saturated at ≥2000 ppm (Borghoff et al., 2017). Thus, the tumorigenicity of ETBE was limited to an exposure concentration that exceeded kinetically derived maximum tolerated dose (Saghir et al., 2010), raising questions on the utility of these findings to human risk assessment.

Genotoxicity of ETBE has been investigated across a variety of test systems (McGregor, 2007; EPA, 2021) and results from these studies were uniformly negative with the following exception. In a series of publications, Weng et al. (2011, 2012, 2013, and 2014) investigated the differential sensitivities of C57Bl6 mice deficient for aldehyde dehydrogenase2 (ALDH2) enzyme using *Aldh2* knockout mice. These studies were conducted to examine the role of oxidative stress and acetaldehyde, an oxidative metabolite, in the genotoxicity of ETBE. Results from these studies seem to suggest that ALDH2 deficient mice ($Aldh+/-$ *and* $Aldh\ -/-$) are slightly more sensitive to ETBE inhalation exposure-related DNA damage than $Aldh2+/+$ mice and that males are more sensitive than females with respect to several genotoxicity endpoints that included oxidative DNA damage and DNA strand breaks. The differences observed between controls and exposed as well as between the genotypes were relatively minor, less than 2-fold in most cases, raising questions on the biological relevance of these statistical findings.

The primary purpose of the current study was to investigate whether ETBE and/or its metabolites can induce mutations in the tumor target tissue following the same route of administration used in the cancer bioassay. If ETBE treatment were to induce oxidative DNA damage (e.g., 8-oxo-dG adducts) or other mutagenic adducts via generation of reactive intermediates during its metabolism, the transgenic assay should be able to detect the resulting mutagenic activity. The lack of a such activity in the tumor target tissue or in a rapidly proliferating tissue indicates that no such reactive metabolites were generated or, if generated, were efficiently detoxified. Currently, transgenic mutation assays are the best available tools to investigate treatment-induced mutagenicity in any tissue of interest. The lack of a mutagenic response in this study adds to the weight of the evidence indicating that DNA reactivity/mutagenicity is unlikely to be the initial key event in the MoA leading to the liver adenomas in the rats.

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Hagiwara et al. (2011 and 2015) investigated the promotional effects of orally administered ETBE on mutagen-initiated tumorigenesis in the rat in a medium-term multi-organ carcinogenesis model. In both studies, ETBE was observed to promote liver carcinogenesis in rats given 1000 mg/kg/day (approximately equivalent to 5000 ppm inhalation exposure) following treatment with a series of mutagens (Hagiwara et al., 2011) or a single mutagen (Hagiwara et al., 2015). No promotional effect was evident at lower exposure doses of 500 mg/kg/day and below indicating a threshold effect for tumor promotion. This observation further confirms that ETBE is not an initiator of liver tumorigenesis and thus is not likely a mutagenic carcinogen. The tumor promotion activity of ETBE is likely related to the induction of cell proliferation as reported by Kakehashi et al. (2013, 2016).

In conclusion, ETBE does not appear to be acting through a mutagenic MoA for liver tumor induction following inhalation exposure. While the available data suggests a nuclear receptor-mediated, non-mutagenic MoA for this carcinogenic effect, further studies are needed to clearly define the dose–response and temporality for the key events along the pathway to the tumor outcome.

AUTHOR CONTRIBUTIONS

B. Bhaskar Gollapudi designed and provided oversight for the study conduct. BG and ER prepared the manuscript.

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The in-life portion of the study was conducted at CRL Laboratories, Ashland, OH (USA) under the Study Directorship of J. T. Weinberg. Processing of tissues for mutation analysis was conducted at BioReliance Corporation, Rockville, MD (USA) under the supervision of Robert Young. The authors would like to thank Marcy Banton for her contribution to the study concept.

CONFLICT OF INTEREST STATEMENT

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