Published in final edited form as:

J Appl Toxicol. 2013 August ; 33(8): 820–827. doi:10.1002/jat.2737.

Methyl tert butyl ether (MTBE) is anti- angiogenic in both *in vitro* and *in vivo* mammalian model systems

John Kozlosky^{*}, Josephine Bonventre[#], and Keith Cooper^{†, #}

^{*}Environmental Science, Rutgers University, New Brunswick, NJ

[#]Joint Graduate Program in Toxicology, School of Environment and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

[†]Department of Biochemistry and Microbiology, School of Environment and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

Abstract

Methyl-tertiary butyl ether (MTBE), a well known gasoline oxygenate, and FDA approved gallstone treatment, has been previously shown to specifically target teleost embryonic angiogenesis. The studies reported here were to determine if similar vascular disrupting effects occurred in higher vertebrate models. Rat brain endothelial cells were isolated and allowed to form microcapillary-like tubes on Matrigel. MTBE (0.34–34.0 mM) exposure resulted in a dose-dependent reduction of tube formation, with the LOAEL at 0.34 mM, while MTBE's primary metabolite, tertiary butyl alcohol had no effect on tube formation. HUVECs, a primary cell line representing macrovascular cells, were able to form tubes on Matrigel in the presence of MTBE (1.25 – 80 mM), but the tubes were narrower than those formed in the absence of MTBE. In a mouse Matrigel plug implantation assay, 34.0 mM MTBE completely inhibited vessel invasion into plugs containing Endothelial Cell Growth Supplement (ECGS) compared to control plugs with ECGS alone. When timed-pregnant Fisher 344 rats were gavaged with MTBE (500–1500 mg/kg) from day 6 of organogenesis through 10 days post parturition, no organ toxicity or histological changes in pup vasculature were observed. Therefore, MTBE is anti-angiogenic at mM concentrations and therefore a potential use as an anti-angiogenic treatment for solid tumors with minimal toxicity.

Keywords

Methyl-tertiary butyl ether; anti-angiogenesis; endothelial cells; developmental study

1. INTRODUCTION

MTBE is best known as an octane booster and oxygenating agent in gasoline, which has resulted in groundwater contamination (Brown, 1997; Gullick and Lechevalier 2000; Post, 2001; Squillace and Moran, 2007; Van Wezel *et al.*, 2009). Workers in the petroleum

industry exposed to MTBE reported a high incidence of neurologic symptoms, including headaches, dizziness, and nausea (Beller and Middaugh, 1992; Moolenaar, *et al.*, 1994; Prah, *et al.*, 1994). In combination with dizziness and nausea, the headaches reported to follow MTBE exposure suggested an effect on the cerebrovasculature (Appenzeller, 1978) and/or an alteration of cranial hemodynamics (Hannerz *et al.*, 1998). Headaches associated with chemical exposures are not uncommon and likely involve similar changes observed following MTBE exposure (Fiedler *et al.*, 2000; Martin and Becker, 1993). MTBE is less well known for its FDA approval use in inoperable gallstone, where it is perfused into the liver biliary track to dissolve the gallstone (Leuschner, 1986; Hellstern *et al.*, 1998). Our studies are the first to examine MTBE's ability to disrupt angiogenesis in multiple systems.

MTBE has been shown to disrupt normal angiogenesis in two embryonic piscine models. In Japanese medaka (*Oryzias latipes*), vasculature fail to develop in embryos treated with 0.11 – 3.4 mM MTBE, while other non-vascular tissues develop normally until vascularization became essential for further growth (Longo, 1995; Kozlosky, *et al.*, 1996). Recently, zebrafish embryos exposed to 0.625–10 mM MTBE were shown to exhibit a dose dependent increase in vascular lesions, including pooled blood in the common cardinal vein, cranial hemorrhages, and abnormal intersegmental vessel formation (Bonventre *et al.*, 2011). These data suggest that MTBE specifically targets the developing vasculature, while having no effect on the development of other organ systems, including the heart. Bonventre *et al.* (2011) reported that the critical period for MTBE toxicity in the zebrafish correlated to an early window of development (15 to 30 hourspost fertilization) where cardiovascular system development is prominent (Kimmel *et al.*, 1995). During the critical period, the expression of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) is significantly decreased. These studies suggested a role for VEGF/VEGFR signaling disruption in MTBE toxicity, which likely results in similar effects in higher vertebrates.

Angiogenesis is the development of blood vessels from pre-existing vessels, and is an integral process in organ growth, repair and solid tumor growth. Angiogenesis primarily occurs during embryonic development, but also occurs in the adult during tissue repair, wound healing, and during aberrant vascular growth in cancer. VEGF is essential to the vasculogenesis, angiogenesis, and hematopoiesis (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). VEGFs are a family of endothelial cell specific growth factors that regulate the proliferation, migration and survival of these cells. VEGFs bind to extracellular portions of tyrosine kinase receptors, VEGFRs. Ligands and receptors are required in assorted combinations for endothelial cell differentiation and migration, but have varying capacities as factors in angiogenesis (Shalaby *et al.*, 1995; Enholm *et al.*, 2001; Habeck *et al.*, 2002). Recently, anti-cancer therapies have included drugs, like Avastin (bevacizumab), that disable tumor cell production and function of VEGFs/VEGFRs to inhibit development of blood vessels simulated by these molecular signals (Dranitsaris *et al.*, 2010).

The unique vascular lesions that occur in piscine embryonic models prompted further investigation into the effect of MTBE on the vasculature of higher vertebrates as a chemical that could be used to disrupt angiogenesis. We hypothesized that because of the highly conserved pathways for angiogenesis in both lower vertebrates and higher vertebrates,

effects seen in angiogenesis in the teleost would be observed in higher vertebrates. Primary cultures of rat brain endothelial cells were evaluated for their ability to form micro-vessels on Matrigel in the presence of MTBE. Tube formation studies were also performed on human umbilical vein endothelial cells (HUVECs), an alternative *in vitro* model of angiogenesis. The mouse Matrigel plug implantation assay was used to determine the effect of MTBE on invasion and neovascularization *in vivo*. A developmental study was performed with pregnant Fisher 344 rats gavaged with MTBE, and the effect on reproductive endpoints on the dam and pups was determined. Studies demonstrated that if high enough concentrations of MTBE were achieved, angiogenesis would be disrupted, but there appeared to be little or no risk to the developing rat pup at concentrations as high as 1500 mg/kg. The results of these studies provide *in vitro* and *in vivo* evidence that MTBE can be used as an effective anti-angiogenic and anti-neovascular therapy in mammalian model systems.

2. MATERIALS AND METHODS

2.1 Chemicals and cell culture supplies

Methyl tertiary butyl ether (MTBE), tertiary butyl alcohol (TBA), sodium heparin, collagenase (type II), percoll, and dextran were purchased from Sigma-Aldrich (St. Louis, MO). M-199 media, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen, Carlsbad, CA). EGM-2 media for human umbilical vein endothelial cell (HUVEC) was purchased from Lonza. Matrigel (phenol red free) and Endothelial Cell Growth Supplement (ECGS) were purchased from BD Biosciences (San Jose, CA). Tissue culture plates were from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ).

2.2 Primary cultures of endothelial cells from rat brain and in vitro angiogenesis

Endothelial cells from the brains of Fisher-344 rats (3 to 4 days old) were isolated by centrifugation on dextrose and percoll density gradients following enzymatic treatment with collagenase type-2, and maintained in culture following the procedures described by Doron, et al. (1991). All procedures were approved through the universities animal care committee. Primary cultures were maintained in Medium 199 containing HEPES (10 mM), L-glutamine (2 mM), 20% FBS, sodium heparin (90 µg/mL), ECGS (20 µg/mL), and 1X penicillin/streptomycin in 24-well culture plates containing glass cover slips precoated with Matrigel. Primary cultures of endothelial cells grown on Matrigel, will elaborate cytoplasmic processes that eventually form capillary-like tubes after two days in culture. A sterile, round, glass cover slip was placed in each well of a twenty-four well culture plate. Each cover slip was then coated with 100 µL Matrigel and the plate was placed at 37°C for 30 minutes to allow the Matrigel to polymerize. Immediately following the isolation procedure, endothelial cells were seeded into the prepared plates at 5×10^4 cells/well. After 48 hours at 37° C in a humidified, 5% CO₂ atmosphere, the treated cells were fixed in 4% paraformaldehyde, immunostained for α-tubulin, and each cover slip was mounted to a microscope slide. Images were acquired from random fields on each slide at 200X magnification. Capillary-like tube formation was quantified by counting the number of tubes in randomly taken photos from each treatment. Statistical significance from control was

determined by Dunnett's Test and the results were expressed as means $\pm$ SEM. A value of $p < 0.05$ was considered statistically significant.

2.3 Human umbilical vein endothelial cell (HUVEC) tube formation assay

HUVECs were obtained from Lonza and cultured in humidified incubators at 37°C with atmospheric air maintained at 5% CO₂. The cells were passaged every four days, and only cells from passages 4–8 were used for the studies. Twenty-four well plates were coated with 100 μ L of Matrigel and allowed to polymerize for 30 minutes. HUVECs were plated at 2.5×10^4 cells/well with 500 μ L of EGM-2 media without serum or supplements. Quadruplicate treatments of 1.25, 2.5, 5.0, and 10.0 mM MTBE, as well as serum negative and serum positive controls were performed on each plate. Concentrations were previously reported to increase vascular lesions in zebrafish (Bonventre et al., 2011). Tube formation was assessed without fixation at 2, 4 and 24 hrs post treatment on an inverted microscope. A second individual scored the plates without knowledge of the treatment to reduce bias. Wells were scored for tube formation at 5 locations: Center, North, South, East and West in each well. Scoring ranged from 0 for no tube formation to 3 for extensive tube network. Average scores for each well were determined and significant difference from control was determined by an ANOVA ($p < 0.05$). Representative pictures were taken of each well at 10X. From these pictures, tube lengths and widths were measured. Length measurements were from the base of one cell projection to another, and width were measure at the calculated mid-way point on the tube. Only tubes for which both ends of the tube were present in the picture were measured. Average lengths and widths were determined for each well and significant difference from control was determined by performing an ANOVA ($p < 0.05$). A second study was performed with 10, 20, 40 and 80 mM MTBE, and wells were scored at 24 hrs as described above.

2.4 Murine Matrigel plug implantation assay for in vivo angiogenesis

The Matrigel plug implantation assay was based on the method of Passaniti, et al. (1992). The assay was performed using two groups of five female mice (C57BL/6NCr Charles River) 6 to 8 weeks of age. A test group was implanted with plugs of Matrigel premixed with 100 ng/mL ECGS to which 34.0 mM MTBE was added. A control group received plugs of Matrigel containing 100 ng/mL ECGS only. The Matrigel (0.5 mL) was injected between the skin and abdominal muscle of each mouse in the groin area close to the dorsal midline. After 10 days, the plugs were dissected from the mice, embedded and sectioned in paraffin, and the sections were stained using hematoxylin-eosin. Angiogenesis was evaluated by observing the invasion of endothelial cells and blood vessels into the Matrigel plug (neovascularization).

2.5 Reproductive vascular development in the rat

MTBE in corn oil was given to four groups of five pregnant female rats (Harlan Sprague-Dawley), orally by gavage, once daily from day 6 of pregnancy through day 10 of gestation at 500, 1000, 1200, or 1500 mg/kg/day. An additional group of five pregnant rats given corn oil alone served as a control. At birth, approximately one half of the animals of each sex from each litter were sacrificed and necropsied, and any gross observations were recorded. Representative samples of brain, lung, liver, kidney, heart, and stomach from these animals

were collected and fixed in 10% neutral buffered formalin. Tissues were processed, sectioned, stained with hematoxylin and eosin, and subsequently examined by light microscopy for deviations in vascular development. The remaining animals from each litter were given MTBE in corn oil orally by gavage once daily from birth through post partum Day 10 at the same level as their respective dam. These animals were then sacrificed on post partum Day 11 and examined as described above. Statistical significance from control was determined by Dunnett's Test and the results were expressed as means $\pm$ SEM. A value of $P < 0.05$ was considered statistically significant. All protocols for the rodent studies were approved through institutional animal care committees.

3. RESULTS

3.1 In vitro rat brain capillary-like tube formation

Isolated endothelial cells from rat brain grown on Matrigel elaborate cytoplasmic processes, which after two days in culture, form microcapillaries (Doron, et al., 1991). The non-treated endothelial cells in our study elaborated cytoplasmic processes that, after two days in culture, formed capillary-like tubes (Figure 1). The length of the tubes was measured using ImageJ software from NIH and there was a significant decrease ($p = 0.01$) in tube length at 0.34 mM and higher concentrations. MTBE (0.34 – 34.0 mM) exposure resulted in a dose-dependent reduction of capillary tube formation (Figure 2). Complete inhibition of rat brain endothelial cell tubes *in vitro* occurred at 34.0 mM MTBE. Treatment with tertiary butyl alcohol (0.34–34.0 mM), a major metabolite of MTBE, had no effect on microcapillary tube formation (data not shown).

3.3 Human umbilical vein endothelial cell tube formation assay

HUVECs are the classic model of *in vitro* angiogenesis due to their ability to rapidly form capillary-like tubes on Matrigel. The effect of MTBE on tube formation over time was determined using concentrations that were previously reported to decrease angiogenesis in zebrafish (Bonventre et al., 2011). Two hours after plating on Matrigel, untreated cells organized into clusters and began to form projections to neighboring endothelial cells (Figure 3A). At 4 hrs, cellular projections in untreated wells were more elaborate, and the basic outline of the tube networks was apparent (Figure 3D), and by 24h, capillary-like networks were elaborate (Figure 3G). MTBE did not inhibit tube formation in the HUVECs as it did in the primary rat brain endothelial cells. No differences were observed in the number of tubes formed with 0.625 to 10 mM MTBE at any time points (Figure 3B, E, H). There was no evidence of cell death or increased loss of cell attachment at any of the times or treatment concentrations (data not shown). At 2 hrs, cells treated with 10 mM MTBE exhibited fewer projections and the arrangement of HUVECs into pre-tube clusters was not as distinct as it was in control (Figure 3C). By 4 h, however, there were no significant differences among the treatments (Figure 3F). Both observers found no significant difference with any concentration of MTBE in the elaborateness of the tube networks at 24 h. In 10 mM wells, the capillary-like tubes appeared to be thinner than the tubes formed in controls (Figure 3I). The average length and width were determined for each well, and the differences were analyzed with an ANOVA. A decrease trend in the lengths ($p < 0.022$) and widths ($p < 0.048$) of tubes among the MTBE treatment groups were observed, however the

all pairwise multiple comparison using the Holm-Sidak method could not determine significant differences between individual concentrations. (Figure 4A, B).

In the second study, the increased MTBE concentrations, 20, 40 and 80 mM, also did not inhibit the formation of a tube network at 24h. However, narrow, skinny tubes were observed in all the treatments, though not quantified. Therefore, MTBE did not inhibit the formation of a capillary-like network in HUVECs at any of the concentrations tested. However, the decreased trend in the length and width characteristics of the tubes indicates that MTBE did affect HUVEC capillary-tube morphology.

3.4 *In vivo* murine Matrigel plug assay

The effect of MTBE on angiogenesis *in vivo* was tested using the Matrigel plug implantation assay (Passaniti, *et al.* 1992). Angiogenesis was assessed by the extent of blood vessel growth at 10 days post implantation into the solid Matrigel plug examined by light microscopy. Matrigel plugs supplemented with ECGS showed a robust angiogenic response (Figure 5A). Small capillaries and vasculature invasion was present throughout the entire control plug. Endothelial cells were lining the capillaries and thin basement membranes were observed. The addition of 34.0 mM MTBE to the ECGS-supplemented plug resulted in near complete inhibition of angiogenesis and small capillary formation (Figure 5B). Only along the periphery were there the beginning capillary formation present, and none were observed penetrating deeper into the plug. There were no lesions or effects on vasculature observed in the surrounding tissue.

3.5 *In vivo* rat developmental study

In an effort to determine if MTBE would affect developing microcapillaries *in utero* and post parturition, pregnant female rats were dosed orally by gavage with MTBE in corn oil at 500 to 1500 mg/kg/day, from implantation (day 6 of pregnancy) through gestation. Pregnant dams receiving 500 or 1000 mg/kg/day MTBE showed no adverse clinical signs; however, those animals receiving 1200 or 1500 mg/kg/day collapsed approximately 30 seconds following dosing and recovered within 2 minutes.

At birth, one half of the rat pups of each sex from each litter were necropsied and any gross observations were recorded. Tissues from these animals were then examined histologically for deviations in vascular development. The remaining animals from each litter were dosed orally by gavage through post partum Day 10 with MTBE in corn oil at the same dose as their respective dam. Narcotic effects, similar to those observed in the dams, were also observed in the pups receiving 1200 or 1500 mg/kg/day MTBE. Control pup body weight gain was not significantly different between any of the treatment groups (Figure 6). Control and treated rat pups were necropsied on post partum Day 11 and examined in a similar manner to that described above. No significant changes in organ weights were observed (Figure 7A–F). In addition, no changes in vascular development were observed in any of the rats following either gross or histological examination at the light microscopy level.

4. DISCUSSION

In the current study, MTBE's anti-angiogenic properties were observed in higher vertebrate rodent models and human cell models. The effects were at concentrations similar (0.34–34.0 mM) to those observed in the fish embryo models, which demonstrated *in vivo* anti-angiogenesis (Bonventre *et al.*, 2010, 2011). The mechanism by which MTBE is inhibiting angiogenesis in the fish embryo model has not been fully elucidated, but in the zebrafish embryo model it has been shown to primarily involve the VEGF/VEGFR pathway and to a lesser extent matrix metalloproteinases (MMP) (Bonventre *et al.*, 2010). VEGF-VEGFR mediated angiogenesis is the primary driver of embryonic vascular development (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Embryonic mice lacking VEGFR-2 have little or no blood vessel formation, suggesting that many downstream effects of VEGF on endothelial cells are mediated through this receptor (Shalaby, *et al.*, 1995). In contrast, mice lacking VEGFR-1 show vascular overgrowth and disorganization (Fong, *et al.*, 1995). The lesions described previously in the developing piscine embryonic vasculature are similar to those reported in the mice lacking VEGFR-2. The inhibition of invasion and neovascularization of the Matrigel plugs containing endothelial cell growth factors suggests that MTBE is disrupting the normal chemical signaling within vascular endothelial cells and/or MMP expression. Exposure of the developing vasculature to MTBE may inhibit VEGFR-2 expression or function, resulting in loss of tyrosine kinase activity and subsequent decreases in VEGFR-2 downstream effects, such as prostacyclin production, cell proliferation, cell migration, nitric oxide production, and cell survival.

MTBE (0.34–34.0 mM) dose dependently reduced capillary-like tube formation in isolated endothelial cells cultured on Matrigel from the brains of 3 to 4 day old Fisher-344 rats (Figure 1 & 2). Significant differences were observed at all the concentrations tested. The lowest concentration tested (0.34 mM) resulted in a 50% reduction and no capillary tubes were observed at 34 mM. The number of attached cells appeared similar across concentrations with no apparent increase in suspended cells. The alpha-tubulin stained photomicrographs demonstrate that as the MTBE concentration increases there is a shortening of pseudopodia formation and loss of tube connection between cells (Yang *et al.*, 1999). The primary endothelial cell cultures were the most sensitive *in vitro* model examined, with a LOAEL of 0.34 mM.

In the study with HUVECs, tube formation was not reduced in the same manner observed in the rat brain endothelial cells; however, the tubes that formed were shorter and thinner than control tubes (Figure 3 & 4). The narrowed width of the HUVEC tubes in 10 mM MTBE mimic the intersegmental vessel lesion that occurs in zebrafish (Bonventre *et al.*, 2011). It is important to note that HUVECs are representative of large vessel endothelial cells since they originate from the umbilical vein, while the rat brain endothelial cells are more representative of microvascular. Macrovascular and microvascular endothelial cells have been shown to express different genes, which may account for the differences in sensitivity to MTBE (Jackson and Ngyun, 1997). However, in both *in vitro* models MTBE disrupted the formation or structure of capillary-like tubes formed on Matrigel, mimicking the vascular effects observed in both fish embryo models.

The murine Matrigel plug assay demonstrated that Matrigel plugs supplemented with 100 ng/mL ECGS showed a robust angiogenic response in 10 day old implants (Figure 5A). This is in stark contrast to the Matrigel plug containing 34.0 mM MTBE added to the ECGS-supplemented plug, which resulted in near complete inhibition of angiogenesis and vascular invasion (Figure 5B). This study demonstrates that MTBE at 34 mM completely inhibits invasion of vasculature *in vivo* in this mouse model. This supports the assumption that if local concentrations of MTBE can reach these levels angiogenesis and vascularization of the tissue would be disrupted in higher vertebrates. The inability to form vascular networks would alter the microenvironment and result in localized ischemia, acidic pH and potential cell death from reduced oxygen tension (Tredan *et al.*, 2007). In both piscine embryonic development studies (Longo, 1995; Bonventre *et al.*, 2011) and the *in vitro* endothelial cell studies described above, exposure to MTBE is continuous over the length of the experiment. Direct contact between MTBE and the vascular tissue and the surrounding connective tissue may be required for anti-angiogenesis to occur.

The developmental toxicity study was carried out to examine if the rodent fetal vasculature would be affected from exposure through the dam. Especially since effects were observed in the fish embryo model and in mice exposed to MTBE via inhalation discussed below. Developmental toxicity from MTBE in lab animals has been reported from inhalation studies only at very high concentrations. Gestational exposure to MTBE showed increased fetal resorption in rats and mice at 2500 ppm, with no effect on the dam (Conaway *et al.*, 1985). However, at 4000 and 8000 ppm, mice exhibited a significant increase in fetal toxicity, post implantation loss, altered sex ratio, craniofacial abnormalities, and skeletal variations, with mild maternal toxicity, while no treatment related toxicity or teratogenicity was observed in rabbits exposed to the same high concentrations (Bevan et al, 1997). In the current rodent. developmental study, no changes in vascular development were observed in any of the rats following either gross or histological examination. There was no change in body weight gain or organ weights following treatment. The lack of an adverse effect could be due to MTBE's rapid metabolism and the elimination through exhalation resulting in a precipitous decrease in circulating MTBE levels (McGregor, 2006). It is also possible that the rat like the rabbit is less sensitive to MTBE than mice. In the fish embryo model assays it was also shown that the Japanese medaka vasculature was much more affected by MTBE than in the Zebrafish. Based on our study there is no evidence that MTBE caused vascular effects on rat pups exposed *in utero* during organogenesis or post partum.

The present studies indicate that MTBE exposure induced alterations in vascular development (rat brain endothelial cells), vascular structure (HUVECs), and vascular invasion (mouse Matrigel plug study). These data also suggest that the developing microvasculature is more sensitive to MTBE toxicity than macrovascular cells. The ability of MTBE to inhibit the invasion and neovascularization in growth factor containing Matrigel plugs in mice suggests a disruption in the signal transduction pathways controlling the proliferation and migration of endothelial cells *in vivo*. Using both *in vitro* and *in vivo* studies, we demonstrated that MTBE vascular toxicity occurs in higher vertebrate model systems. Our data suggest that MTBE can be used as a model anti-angiogenic compound. MTBE can also be used as a tool to understand the differences in responses to toxic insult

between microvascular and macrovascular populations of endothelial cells. MTBE is currently FDA approved for therapy for dissolving cholesterol gallstones in inoperable patients, with minimal side effects reported following the procedure (Leuschner, 1986; Hellstern *et al.*, 1998). Since tumor growth is greatly dependent on the development of a neovascular network, MTBE should be considered as a potential vascular-disrupting agent that could be used in combination with hypoxia activated drugs (Ferrara and Kerbel 2005). The potential for using MTBE's vascular disrupting capabilities for shrinking solid tumors in the clinical setting alone or in combination with other treatments and the mechanism by which this occurs should be researched further.

Acknowledgments

We would like to thank Dr. Ken Reuhl for his early support of this project, Dr. Lori White and her lab for assistance and equipment, and Bristol Myers Squibb for allowing portions of this research to be carried out on site.

Funding: This work was supported by New Jersey Agricultural Experiment Station (NJ01201) through Cooperative State Research, Education and Extension Services, the National Institute of Environmental Health Sciences (ES07148), the Environmental and Occupational Health Sciences Institute (ES05022), the New Jersey Water Resources Research Institute (2010NJ198B), New Jersey Department of Environmental Protection, Division of Science, Research and Technology (SR09-019).

References

- Appenzeller O. Cerebral aspects of headache. *Med Clin N Amer.* 1978; 62(3):467–480. [PubMed: 351308]
- Beller, HR.; Middaugh, JP. Potential illness due to exposure to oxygenated fuels: Fairbanks, Alaska. Conference on MTBE and Other Oxygenates: A Research Update; 1992.
- Bevan C, Tyl RW, Neeper-Bradley TL, Fisher LC, Panson RD, Douglas JF, Andrews LS. Developmental toxicity evaluation of MTBE by inhalation in mice and rabbits. *J Appl Toxicol Suppl.* 1997; 1:S21–29.10.1002/(SICI)1099-1263(199705)17:1+<S21::AID-JAT407>3.0.CO;2-E
- Bonventre, JA.; White, LA.; Cooper, KR. Increased lesion occurrence and decreased gene expression following embryonic exposure to MTBE, ETBE, and TAME in the zebrafish (*Danio rerio*). MASOT; Woodbridge, NJ: 2010.
- Bonventre JA, White LA, Cooper KR. Methyl tert butyl ether targets developing vasculature in zebrafish (*Danio rerio*) embryos. *Aquatic Toxicol.* 2011; 105:29–40.10.1016/j.aquatox.2011.05.006
- Brown S. Atmospheric and potable water exposure to methyl tert-butyl ether. *Reg. Tox. Pharm.* Environmental Toxicology. 1997; 25:256–276.10.1002/tox
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Naggy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996; 380:436–438.10.1038/380435a0
- Conaway CC, Schroeder RE, Snyder NK. Teratology evaluation of MTBE in rats and mice. *J Toxicol Environ Health.* 1985; 16(6):797–809. [PubMed: 4093995]
- Doron D, Jacobowitz D, Heldman E, Feuerstein G, Pollard H, Hallenbeck J. Extracellular matrix permits the expression of von willebrand's factor, uptake of di-i-acetylated low density lipoprotein and secretion of prostacyclin in cultures of endothelial cells from rat brain microvessels. *In Vitro Cell Dev Biol.* 1991; 27A:689–697.10.1007/BF02633213 [PubMed: 1917789]
- Dranitsaris G, Edwards S, Edwards J, Leblanc M, Abbott R. Bevacizumab in combination with folfirchemotherapy in patients with metastatic colorectal cancer: an assessment of safety and efficacy in the province of Newfoundland and Labrador. *Curr Oncol.* 2010; 17(5):12–6.10.1186/1471-2407-9-347 [PubMed: 20975873]
- Enholm B, Karpanen T, Jeltsch M, Kubo H, Stenback F, Prevo R, Jackson DG, Yla-Herttuala S, Alitalo K. Adenoviral expression of vascular endothelial growth factor-c induces

- lymphangiogenesis in the skin. *Circulation Research*. 2001; 88:623–629.10.1161/01.RES.88.6.623 [PubMed: 11282897]
- Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powel-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of VEGF gene. *Nature*. 1996; 308:439–442.10.1038/380439a0 [PubMed: 8602242]
- Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005; 438:967–974.10.1038/nature04483 [PubMed: 16355214]
- Fiedler N, Kelly-McNeil K, Mohr S, Lehrer P, Opiekun RE, Lee C, Wainman T, Hamer R, Weisel C, Edelberg R, Liroy PJ. Controlled human exposure to methyl tertiary butyl ether in gasoline: symptoms, psychophysiologic and neurobehavioral responses of self-reported sensitive persons. *Environ Health Perspect*. 2000; 108:753–763. [PubMed: 10964796]
- Fong G-H, Rossant J, Gertsenstein M, Breitman M. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995; 376:66–70.10.1038/376066a0 [PubMed: 7596436]
- Gullick RW, Lechevalier MW. Occurrence of MTBE in drinking water sources. *J American Water Works Assoc*. 2000; 92(1):100–113.
- Hannerz J, Jogestrand T. Is chronic tension-type headache a vascular headache? The relation between chronic tension-type headache and cranial hemodynamics. *Headache*. 1998; 38(9):668–75.10.1046/j.1526-4610.1998.3809668.x [PubMed: 15613178]
- Habeck H, Odenthal J, Walderich B, Maischein H, Schulte-Merker S. Analysis of zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr Biol*. 2002; 12(16):1405–1412.10.1016/S0960-9822(02)01044-8 [PubMed: 12194822]
- Hellstern A, Leuschner U, Benjaminov A, Ackermann H, Hein T, Festi D, Orsini M, Roda E, Northfield TC, Jazrawi R, Kurtz W, Schmeck-Lindenau HJ, Stumpf J, Eidsvoll BE, Aasland E, Luc G, Boehnke E, Wurbs D, Delhave M, Cremer M, Sinn I, Horing E, vGaisberg U, Neubrand M, Paul F, et al. Dissolution of gallbladder stones with methyl tert-butyl ether and stone recurrence: a European survey. *Dig Dis Sci*. 1998; 43(5):911–20.10.1002/hep.1840190122 [PubMed: 9590398]
- Jackson CJ, Nguyen M. Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. *Int J Biochem Cell Biol*. 1997; 29(10):1167–77.10.1016/S1357-2725(97)00061-7 [PubMed: 9438380]
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995; 203(3):253–310. [PubMed: 8589427]
- Kozlosky J, Longo S, Cooper K, Philbert MA. Comparative studies of the acute toxicity of mtbe in japanese medaka and cultured rat brain microvessels. *Fundamental and Applied Toxicology*. 1996; 30 (Suppl):193. Abstract.
- Leuschner U. Endoscopic therapy of biliary calculi. *Clin Gastroentero*. 1986; 15:333–358.
- Longo, SE. MS Thesis. Rutgers University, Graduate School; New Brunswick: 1995. Effects of methyl tert-butyl ether and naphthalene on the embryo of the Japanese medaka.
- Martin RW, Becker C. Headaches from chemical exposures. *Headache*. 1993; 33(10):555–559.10.1111/j.1526-4610.1993.hed3310555.x [PubMed: 8294194]
- McGregor D. Methyl tertiary-Butyl Ether: Studies for Potential Human Health Hazards. *Crit Rev in Toxicol*. 2006; 36(4):319–358.10.1080/10408440600569938 [PubMed: 16809102]
- Moolenaar R, Hefflin B, Ashley D, Middaugh J, Etzel R. Methyl tertiary butyl ether in human blood after exposure to oxygenated fuel in Fairbanks. *Alaska Arch Environ Health*. 1994; 49:402–409.10.1080/00039896.1994.9954993
- Passaniti A, Taylor R, Pili R, Guo Y, Long P, Haney J, Pauly R, Grant D, Martin G. Methods in laboratory investigation: a simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Inv*. 1992; 67:519–528.
- Post, G., editor. MTBE in New Jersey's Environment. New Jersey Department of Environmental Protection; 2001.
- Prah J, Goldstein G, Devlin R, Otto D, Ashley D, House S, Cohen K, Gerrity T. Sensory, symptomatic, inflammatory, and ocular responses to and the metabolism of methyl tertiary butyl

ether in a controlled human exposure experiment. *Inhal Toxicol.* 1994; 6(6):521–538.10.3109/08958379409003038

Shalaby F, Rossant J, Yamaguchi T, Gertsenstein M, Wu X-F, Breitman M, Schuh A. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature.* 1995; 376:62–66.10.1038/376062a0 [PubMed: 7596435]

Squillace PJ, Moran MJ. Factors associated with sources, transport, and fate of volatile organic compounds and their mixtures in aquifers of the United States. *Environ Sci Technol.* 2007; 41:2123–2130.10.1021/es061079w [PubMed: 17438752]

Tredan O, Galmararini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. *JNCI.* 2007; 99(19):1441–1454.10.1093/jnci/djm135 [PubMed: 17895480]

United States DOE-EAI. [December 2010] Monthly MTBE production, updated 12/30/10. 2009. http://tonto.eia.doe.gov/dnav/pet/pet_pnp_oxy_dc_nus_mbb1_m.htm

Van Wezel A, Puijker L, Vink C, Versteegh A, de Voogt P. Odour and flavor thresholds of gasoline additives (MTBE, ETBE and TAME) and their occurrence in Dutch drinking water collection areas. *Chemosphere.* 2009; 76(5):672–676.10.1016/j.chemosphere.2009.03.073 [PubMed: 19477481]

Yang S, Graham J, Kahn JW, Schwartz EA, Gerritsen ME. Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumenformation in three-dimensional collagen gels. *Am J Pathol.* 1999; 155(3):887–95.10.1016/S0002-9440(10)65188-7 [PubMed: 10487846]

Figure 1. MTBE inhibited *in vitro* endothelial tube formation: The formation of capillary-like structures by isolated rat brain endothelial cells grown on Matrigel was significantly attenuated in a dose-dependent manner following treatment with MTBE for 48 hours. Representative photographs (200X) of (A) untreated rat brain endothelial cells seeded on Matrigel-coated glass cover slips after 48 hours and following treatment with (B) 0.34 mM MTBE, (C) 3.40 mM MTBE, or (D) 34.0 mM MTBE for 48 hours. Cells were fixed in 4% paraformaldehyde and immunostained for α -tubulin.

Figure 2. Rat brain endothelial cell tube formation following 48 hr treatment with MTBE. ** $P < 0.01$ relative to control.

Figure 3. Representative pictures of HUVEC tube formation time course assay. Untreated control cells A, D, G show a clear progression of tube formation from preliminary organization on the Matrigel at 2 hrs to an elaborate network of capillary-tubes at 24 hrs. MTBE treatments are representative of the lowest (0.625 mM: B, E, H) and highest (10 mM: C, F, I) concentrations used for the study.

Figure 4.

The effect of MTBE on HUVEC tube morphometrics. At 24hrs, there was no significant difference in the number of tubes per well for any treatment group. There was a significant decreased trend in the length of tubes formed (A) and tube widths (B). Tubes were measured with the Adobe Photoshop measurement tool, and the units are relative. Tube length was measured from the base of one cell projection to another, and the width was the half-way point on the tube. Only tubes for which both ends of the tube were present were measured. The significant trend was determined by One-way Analysis of Variance ($p < 0.05$), but the Holm-Sidak pairwise comparison ($p < 0.05$) was not significant for any treatment for either length or width.

Figure 5.

Representative plugs from the murine Matrigel plug implantation assay: Matrigel plugs containing the indicated amount of MTBE and/or ECGS were embedded between the skin and abdominal wall of mice for 10 days, then collected and sectioned. (A) Matrigel plugs supplemented with 100 ng/mL ECGS showed a robust angiogenic response. (B) The addition of 34.0 mM MTBE to the ECGS-supplemented plug resulted in near complete inhibition of angiogenesis. The images are hematoxylin and eosin stained cross sections of the plugs at the boarder of the skin at 100X magnification.

Figure 6. Body weights (mean of 47–53 pups per time point) in rat neonates from birth (Day 1) through Day 10.

Figure 7.

Tissue weights (mean of 22–29 pups per time point) in rat neonates. Tissue weights include liver (A), brain (B), kidney (C), stomach (D), lung (E), and heart (F) from Day 1 (birth) and Day 11.