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Toxicology

Responses of the steroidogenic pathway from exposure to methyl-tert-butyl ether and tert-butanol

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ABSTRACT

Methyl tertiary-butyl ether (MTBE) is a solvent and fuel additive included in reformulated gasoline to increase combustion efficiency. While widespread use in motor fuels in the U.S. was discontinued after MTBE was detected in surface and ground waters due to concerns about environmental persistence and water quality, it is still manufactured in the U.S. for export. Questions concerning the etiology of rat Leydig cell and mouse liver tumors identified in extremely high dose cancer studies have led to an interest in evaluating potential hormonal imbalances and endocrine system involvement. To address the possibility that MTBE or its metabolite, tert-butanol (TBA), are interacting with components of the endocrine system that are involved in steroidogenesis a number of targeted experiments were performed focusing mostly on the primary gonadal steroids, estradiol and testosterone. The goal of the experiments was to gain a better understanding of potential interactions with the steroidogenic pathway, including effects specifically on aromatase, the P450 enzyme that converts testosterone to estradiol. In three GLP-compliant in vitro guideline studies, MTBE and TBA were classified as non-binders to the androgen receptor, were classified negative for effects on testosterone and estradiol in the steroidogenesis assay, and were classified as non-inhibitors of aromatase activity. In three 14-day in vivo experiments involving gavaging of male Sprague-Dawley rats with doses of MTBE ranging from 400 to 1500 mg/kg bw/day, the lack of definitive and consistent supporting statistically significant findings in steroid hormone measurements and aromatase activity and mRNA measured in liver and testis microsomes further suggested that it is unlikely that MTBE is interacting with the endocrine system directly. Evidence of other underlying systemic effects were also seen, including reduced body weight gain, increased adrenal weights, and elevated corticosterone suggestive of a more general stress response. Taken together, the results from these studies suggest that MTBE and TBA do not directly impact the steroidogenic pathways involved in estrogen and androgen production.

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1. Introduction

Methyl tertiary-butyl ether (MTBE) is a solvent and fuel additive included in reformulated gasoline to increase combustion efficiency. Widespread use in motor fuels in the U.S. was discontinued after MTBE was detected in surface and ground waters because of concerns about environmental persistence and water quality. MTBE

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is still used elsewhere as a fuel oxygenate (CONCAWE, 2012), and manufacturing in the U.S. continues for export.

MTBE is readily absorbed orally and by inhalation, but has a very low rate of dermal absorption (ATSDR, 1996; Prah et al., 2004; McGregor, 2006). Plasma half-life is short, ranging from 0.45 to 0.79 h in adult male rats exposed by ingestion, inhalation or i.v. administration (Miller et al., 1997). T-butanol (tert-butyl alcohol; TBA) is a major metabolite of MTBE (Miller et al., 1997). Toxicokinetics studied in human volunteers inhaling MTBE show a triphasic plasma elimination curve with three half-lives on the order of 9–12 min, 1.4–1.5 h and 17–21 h, depending on air concentration (Johanson et al., 1995). Acute toxicity is low based on lethality

studies conducted in rats: oral LD50 of approximately 4 g/kg bw, dermal LD50 of greater than 10 g/kg bw, and inhalation LC50 of 120 mg/L (ARCO, 1980). Transient sedation and ataxia observed after high doses are typical of effects seen with MTBE and other ethers.

With exposure to high concentrations of MTBE, far in excess of any relevant human exposure, an increased tumor incidence has been reported in some of the MTBE rodent cancer bioassays (Belpoggi et al., 1995; Bird et al., 1997). Both the International Agency for Research on Cancer (IARC) and the European Union have reviewed the available cancer studies and have concluded that MTBE is not classifiable as to human carcinogenicity (IARC, 1998) or is a borderline case between no-classification and the lowest cancer category (Cat 3) referring to insufficient evidence for humans (EU Risk Assessment, 2002). MTBE is not considered to be genotoxic, and because the tumors reportedly observed in rodent studies are species- and gender-specific, alternative mechanisms are suggested, including an endocrine imbalance (ATSDR, 1996; Bird et al., 1997; McGregor, 2006; Cruzan et al., 2007).

Impacts on endocrine system endpoints have been noted in several subchronic rodent toxicology studies using repeat high MTBE doses comparable to those used in the cancer bioassays. For example, most relevant to the studies discussed in the present paper are several earlier reports of reduced circulating testosterone levels when male rats are dosed by gavage for up to 1500 mg/kg/day for 14–28 days (Williams et al., 2000; de Peyster et al., 2003; Li et al., 2008). When MTBE is added to freshly isolated rat Leydig cells, decreased testosterone production is observed (de Peyster et al., 2003). Relatively high concentrations in the 50–100 millimolar range were used in that experiment showing statistically significant inhibition. Because cytotoxicity was not measured in these experiments, the possibility that a reduction in viable cells could explain all or even part of the testosterone reduction seen in cell supernatants must be considered.

Effects on steroid hormones are not necessarily a result of a direct interaction with the endocrine system. Induction of total liver cytochrome P450 and increased activity of some specific liver P450 isozymes involved in testosterone metabolism have been measured following high doses of MTBE (Savolainen et al., 1985; Williams and Borghoff, 2000; de Peyster et al., 2003). In addition, de Peyster et al. (2003) reported weight loss in experiments where a reduction in testosterone was noted which could account for the observations in the rats (Bergendahl et al., 1989). General stress causing elevated levels of corticosterone can also play a role in altering hormone concentrations (Monder et al., 1994). In a number of reported experiments, final serum levels of steroid hormones were assessed a short time after the final dose of MTBE was administered, during which ataxia and other overt signs of toxicity were noted (Williams et al., 2000; de Peyster et al., 2003).

To address the possibility of MTBE or its metabolite, TBA, interacting with steroidogenesis which could result in hormonal imbalance a number of experiments were performed. The goal of the three *in vitro* experiments was to gain a better understanding of the interaction of MTBE and its metabolite, TBA, on the steroidogenic pathway, including effects specifically on aromatase, the P450 enzyme that converts testosterone to estradiol. *In vitro* approaches were used to address binding of MTBE and TBA to the androgen receptor and their potential effects on androgen or estrogen steroid production, as well as activity of the enzyme aromatase. Three *in vivo* studies were also performed to evaluate the effects of MTBE on gonadal and also adrenocortical steroid hormone production, aromatase and cytochrome P450 activity, as well as clinical chemistry measures and renal toxicity biomarkers for a better understanding of general systemic toxicity occurring at the doses used.

2. Materials and methods

2.1. In vitro studies

The three *in vitro* studies were performed at CeeTox, Inc. Kalamazoo, MI. All three were conducted in compliance with the U.S. EPA Good Laboratory Practice regulations, Part 160, with the exception that the purity and concentrations of test and control substances were not analytically verified by the laboratory during the study.

Chemicals. The test chemicals MTBE (CAS No.1634-04-4, purity 99.9%) and tert-butanol (TBA) (CAS No. 75-65-075-65-0, purity 99.9%) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was selected as a suitable vehicle for use in all three studies.

For the ARB study, the reference compound R1881 (methyltrienolone, CAS No. 965-93-5. Purity 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The weak positive control dexamethasone (CAS No. 50-02-2, purity 98%) was purchased from Sigma-Aldrich (Buchs, Switzerland). The radioligand [^{3}H]-R-1881 was purchased from Perkin-Elmer (Boston, MA, USA) and had a specific activity of 82.7 Ci/mol for the first two independent runs completed within a two-day period, and a specific activity of 82.5 Ci/mol for the third run ten days later.

For the steroidogenesis study, the positive control compounds forskolin (CAS No. 66575-29-9, purity 98%) and prochloraz (CAS No. 67747-09-5, purity 99.1%), as well as 22R-hydroxycholesterol (CAS No. 17954-98-2, purity 99.0%) were also purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

For the aromatase study, the positive control 4-hydroxyandrostendione (4OH-ASDN, formestane, purity 99.6%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Non-radiolabeled 4-androstene-3,17-dione (ASDN, purity 99.8%) was purchased from Steraloids, Inc (Newport, RI, USA) and radiolabeled [1β-^{3}H]-androstenedione ([^{3}H]-ASDN, purity >97%, specific activity 26.3 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA, USA).

2.1.1. Androgen receptor binding

The methodology to evaluate the ability of the test substances to interact with the androgen receptors (ARs) isolated from rat prostates followed the U.S. EPA OPPTS 890.1150 guideline (U.S. EPA, 2009a). Prostate glands from 90-day old (<1 day since castration) male Sprague-Dawley rats were purchased from Charles River Laboratories (Run 1, Portage MI; Runs 2 and 3, Raleigh, NC). Cytosol was prepared and verified according to EPA guideline OPPTS 890.1150. MTBE and TBA solutions were prepared fresh on the day of the assay for all three valid independent runs with a maximum concentration of 10^{-3} M, the limit concentration for the assay. Solubility of the test solutions was assessed by laser based light scattering using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany). All test and control substances were prepared in DMSO limiting the final concentration of the solvent in the assay medium to ~3.2% (v/v).

The positive control, R1881, and weak positive control, dexamethasone, were prepared in DMSO (~3.2% (v/v)) on the day of the first independent run and aliquots were frozen for use in the second and third valid runs. The positive control, R1881, strongly binds ARs and was included, along with the weak positive control, to ensure that the run was properly performed and to allow an assessment of variability in the conduct of the assay across time. Final concentrations of unlabeled R1881 ranged from 3.1×10^{-6} to 3.1×10^{-11} M. The concentration range tested for the weak positive control was from 1×10^{-3} to 1×10^{-10} M.

All stock and buffer solutions were prepared according to EPA OPPTS 890.1150 guidelines (U.S. EPA, 2009a). A 60% hydroxyapatite (HAP) slurry, washed three times, was prepared one day before use for each of the three independent runs. After the third wash, the HAP slurry settled overnight at approximately 4 °C. On the day of use, the supernatant was removed and the HAP was resuspended to a final volume of 60% HAP and 40% cold 50 mM Tris buffer.

[^{3}H]-R1881 was prepared on the day of assay. Before performing dilutions, the specific activity was adjusted for decay over time to obtain a final concentration of 1 nM in the buffer solution.

Following the OPPTS guideline, [^{3}H]-R1881 and triamcinolone acetonide were added to all tubes. For the 3 tubes at the beginning of assay and at the end of assay, 100× inert R1881 (30 μL of 1 μM) was also added. These were the non-specific binding (NSB) tubes. The tubes were placed in speed-vac and dried. An aliquot of cytosol was thawed on ice and diluted, if necessary, to the pre-determined optimal protein concentration. For the assay tubes, 10 μL of each concentration of test substance and control were added, followed by 300 μL of the thawed cytosol. The temperature of the tubes and contents were kept at approximately 4 °C prior to the addition of the cytosol. The assay tubes were vortexed after additions and incubated at approximately 4 °C for 16–20 h on a rotator.

To separate the bound and free [^{3}H]-R1881, the AR assay tubes were removed from the rotator and placed in an ice-water bath. 100 μL of each incubation tube was transferred to tubes containing ice cold HAP slurry (60% in 50 mM Tris buffer). The tubes were vortexed for approximately 10 seconds at ~5 min intervals for a total of ~20 min with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, Tris buffer was added, the tubes were quickly vortexed, and centrifuged at approximately 4 °C for approximately 3 min at $700 \times g$. After centrifugation, the supernatant containing the free [^{3}H]-R1881 was decanted immediately