Subchronic Studies in Sprague-Dawley Rats to Investigate Mechanisms of MTBE-Induced Leydig Cell Cancer

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High MTBE exposures caused rat Leydig cell (LC) tumors in inhalation and gavage cancer bioassays. Investigating early endocrine changes consistent with known mechanisms of LC carcinogenesis, we gavaged adult male Sprague-Dawley rats with MTBE in five different subchronic experiments and studied testosterone biosynthesis in isolated rat LCs exposed in vitro to MTBE or a major metabolite, t-butanol. In vitro LC testosterone production declined 29–50% following 3-h exposures to 50–100 mM MTBE or t-butanol. Within hours after gavaging with 1000 or 1500 mg/kg MTBE, circulating testosterone declined to 38–49% of control (p < 0.05). If sampled longer after treatment or with lower doses, testosterone reductions were less dramatic or nondetectable even after 28 days of treatment. Accessory organ:brain weight ratios decreased only slightly although showing dose response with 40–800 mg/kg/day after 28 days. High MTBE doses caused slight liver weight and total P450 increases. Reduced aromatase activity in liver and testis microsomes predicted lower serum estradiol, but estradiol was 19% higher than corn oil controls concurrent with testosterone reduction 1 h after the last of 14 daily 1200-mg/kg estradiol was 19% higher than corn oil controls concurrent with testosterone reduction 1 h after the last of 14 daily 1200-mg/kg doses (p < 0.05). Pituitary luteinizing hormone (LH) and prolactin measured in both intact and orchietomized rats, with testosterone implants in some castrated rats providing stable levels of testosterone, revealed no consistent direct effect on hypothalamic-pituitary function. MTBE-treated rat livers showed no evidence of peroxisome proliferation, a characteristic of some LC carcinogens. Considering recognized mechanisms of Leydig cell cancer in rats, collectively these results suggested reduced LC steroidogenesis enzyme activity as a possible mechanism underlying MTBE LC carcinogenesis.

Key Words: Methyl t-butyl ether (MTBE); rats; mechanisms; Leydig cell cancer; endocrine disruption.

Methyl tertiary-butyl ether (MTBE) is used as a solvent and motor fuel oxygenate. Inhalation, dermal absorption, and ingestion of contaminated water are all possible routes of exposure (ATSDR, 1996). MTBE has low acute toxicity and an oral LD50 of approximately 4 g/kg in rats. It is metabolized mainly to t-butanol and formaldehyde (Miller et al., 1997). Ether-like CNS effects observed in laboratory animals given high doses include transient sedation and ataxia (ATSDR, 1996). Acute exposures can usually be avoided, but health concerns remain about environmental persistence and increased species- and gender-specific tumor incidences in rodent cancer bioassays, although at doses far exceeding typical human exposures.

Statistically increased Leydig cell adenomas were observed in Fischer 344 rats inhaling 3000 and 8000 ppm MTBE for 24 months (Bird et al., 1997); however, the control group tumor incidence used for comparison was unusually low. Another cancer bioassay in Sprague-Dawley rats gavaged with 1000 mg/kg MTBE in olive oil four times a week for two years also resulted in a higher incidence of Leydig cell tumors (Belpoggi et al., 1995). Longer lifespan of the animals receiving this dose relative to controls complicates interpretation of this study, since increases in Leydig cell tumors are also associated with longer survival rates (Capen, 2001). Assuming MTBE was responsible for increased Leydig cell tumor incidence in these cancer bioassays, the main objective addressed by our studies reported here was to elucidate how this might have occurred.

Lack of significant genotoxicity (ATSDR, 1996) and evidence suggestive of endocrine modulation in mice led us to initiate a series of experiments focusing on the effects of high doses of MTBE on the male rat endocrine system. An unusual increase in liver adenomas observed along with decreased uterine cystic hyperplasia in CD-1 mice given chronic high doses of MTBE (Bird et al., 1997), followed up by studies to elucidate the mechanism of these female mouse liver tumors (Moser et al., 1996, 1998), all suggested a mechanism involving hormone disruption that is not mediated through estrogen receptors but is possibly related to altered steroid metabolism. When we began the present series of experiments, others had also shown that MTBE induces P450 in male rat liver (Brady et al., 1990). Possible consequences of hepatic P450 induction include increased testosterone catabolism, a compensatory increase in luteinizing hormone (LH) secretion, chronic over-stimulation of Leydig cells, and ultimately Leydig cell hyperplasia and neoplasia. In initial experiments we and others observed some effects of MTBE on endocrine activity in male...
rats (Day et al., 1998; Williams et al., 2000) and induction by MTBE of selected P450 isozymes involved in testosterone metabolism (Williams and Borghoff, 2000), although the underlying mechanism of the Leydig cell tumors remained to be elucidated.

Many rat Leydig cell carcinogens increase LH and stimulate Leydig cells by various mechanisms. If prolonged, this can increase mitosis and benign focal hyperplasia progressing to malignancies (Clegg et al., 1997; Cook et al., 1999). Because rat Leydig cell physiology is not identical to human, only some mechanisms of rat Leydig cell carcinogenesis appear to be relevant to humans, and rat Leydig cell responsiveness to xenobiotics is often acknowledged to be an unreliable predictor of effects in humans (Clegg et al., 1997; Cook et al., 1999).

Like Williams et al. (2000) we hypothesized that Leydig cell tumors observed after prolonged MTBE exposure may result from chronic disruption in normal hypothalamic-pituitary-gonadal and/or liver functions. Five separate in vivo experimental protocols using Sprague-Dawley rats are reported here, each with different objectives related to the central question: Could MTBE cause rat Leydig cell tumors by altering hormone levels? Effects of MTBE and its main metabolite, t-butanol, on testosterone production were also studied in isolated rat Leydig cell cultures. Evidence of testosterone biosynthesis inhibition and other effects seen after subchronic gavage exposure with MTBE doses comparable to those used in carcinogenesis bioassays were then compared with patterns of effects of other rat Leydig cell carcinogens having recognized mechanisms of action. Specific rat Leydig cell mechanisms considered were androgen receptor antagonism and testosterone biosynthesis, aromatase, or 5α-reductase inhibition. Evidence of peroxisome proliferation was also examined because a number of peroxisome proliferators also cause rat Leydig cancer. Involvement of other organs outside of the hypothalamic-pituitary-testicular axis that impact on axis function, specifically adrenal and thyroid glands, was also considered.

MATERIALS AND METHODS

Chemicals. MTBE (CAS No. 1634-04-4) was spectrophotometric grade with purity reported as 99.8–99.9%. (Burdick & Jackson/I. T. Baker). Other reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted.

Animals. Adult Sprague-Dawley male rats were obtained from Harlan (San Diego, CA) or B&K (Richmond, CA; Experiment 2). The oral route of MTBE exposure was the focus of our experiments, and this strain had developed Leydig cell tumors after gavage exposure (Belpoggi et al., 1995). All husbandry and treatment procedures were approved by the University Animal Subjects Committee (IACUC). Standard rat diet 5001 (Purina Mills, Inc.) and tap water were available ad libitum. Rats were caged in pairs in polycarbonate box cages with hardwood bedding (Sani-Chips, Sani-Pure, SaddleBrook, NJ). Animals were randomly assigned to treatment groups after acclimation to the vivarium environment for at least two weeks. Statistical analysis of body weights indicated no significant differences among the groups at the time of assignment to dose groups. Acclimation included routine handling and gavaging to minimize these potentially confounding sources of stress.

Treatments, hormone and tissue sampling in all in vivo experiments. MTBE was given by gavage in corn oil at the same time every day of an experiment. MTBE is well absorbed from the gastrointestinal tract and has a plasma half-life of 30–60 min (Miller et al., 1997). Dose selection varied across experiments depending on objectives and information available at that time and is explained with other protocol details below. Body weights were recorded regularly several times each week, at which time doses were adjusted for body weight. Rats were sacrificed by CO2 asphyxiation (Experiments 1 and 4) or by decapitation if a large volume of trunk blood was desired for multiple hormone analyses (Experiments 2, 3, and 5). When blood was drawn for hormone analyses at times other than at sacrifice (Experiments 1, 2, and 3), then the tail blood volume targeted ranged from 0.5 to 2.0 ml per animal, depending on the number and types of analyses planned. Serum in Experiments 1 and 4 was obtained by collecting blood in vacutainers without anticoagulant or other preservative, allowing to clot for an hour, then centrifuging for 15 min at 1200 × g. In Experiments 2, 3, and 5, blood was collected in heparinized tubes, placed on ice, and centrifuged at 2800 × g for 15 min to separate plasma from erythrocytes. Aliquots for hormone measurements were analyzed immediately or stored at −70°C until analysis. Wet organ weights were recorded. Seminal vesicles were weighed without expression of fluid.

Objectives and Designs of in Vivo Studies

Experiment 1. This was an exploratory study investigating the relationship of P450 enzyme induction and steroid metabolism. The primary objective was to determine whether high gavage doses of MTBE, comparable to those used in the gavage carcinogenesis assay (Belpoggi et al., 1995), would induce liver P450 concurrent with reduced circulating testosterone levels. The possibility that corticosterone could have played a role in causing the Leydig cell cancers was also explored because increased stimulation of testicular glucocorticoid receptors impairs steroidogenesis (Monder et al., 1994). Elevated adrenal weights or high serum corticosterone had been reported in several other studies involving male rats treated with MTBE (Bird et al., 1997; Lington et al., 1997; Williams et al., 2000). Matched group mean body weights at the start of exposure ranged from 383–386 g. MTBE treatments administered every other day starting on day 1 consisted of 1000 mg/kg or 1500 mg/kg in corn oil, or corn oil vehicle (n = 12). The 1000 mg/kg dose was chosen because this had resulted in increased Leydig cell tumors after two years of gavage for four times a week (Belpoggi et al., 1995). The 1500 mg/kg dose was adopted from Robinson et al. (1990), the only other MTBE study using gavage that was readily available at the time Experiment 1 was conducted. An untreated control group (n = 12) was also included, but untreated and corn oil control values were ultimately pooled. Because of weight loss in MTBE-treated animals and one death, the 1000 mg/kg dose was reduced to 500 and the 1500 mg/kg dose was reduced to 750 mg/kg every other day starting with day 13. A total of 14 gavage treatments were administered over 27 days. Tail blood samples were obtained for measurement of serum testosterone in some animals 4–5 h after the first treatment, and in all rats the morning after the afternoon dose administration on day 13 treatment (i.e., day 14). Testosterone was measured again in terminal blood samples obtained by cardiac puncture after sacrifice, which occurred on day 28, 16–20 h after the final MTBE treatment. Corticosterone was measured in remaining serum when sample volume was sufficient. Organs were weighed for evidence of gross effects, including androgen insufficiency. Liver microsomes were prepared by differential centrifugation for measurement of total protein (Coomassie Plus, Pierce Chemical, Rockford, IL) and total P450 (Omura and Sato, 1967).

Experiment 2. This study was designed to gain a better understanding of the mechanisms underlying hormonally-mediated responses seen in Experiment 1 using more frequent but less acutely toxic doses than were used initially. Pituitary hormones (LH, prolactin) were sampled in addition to testosterone and corticosterone, and the impact of MTBE on weights of other endocrine organs, (pituitary, thyroid, adrenals) that can affect testicular and hepatic functions was also examined. Four treatment groups consisted of daily MTBE doses of 40, 400, or 800 mg/kg in corn oil or corn oil vehicle only (n =
12–13). A then recently published pharmacokinetics study (Miller et al., 1997) characterized oral MTBE pharmacokinetics using single 40 and 400 mg/kg doses. According to that report, MTBE metabolism initially to t-butanol and subsequently to other metabolites is saturated at an oral dose of 400 mg/kg, at which point the elimination half-life of MTBE is about 47 min. The 800 mg/kg dose was slightly lower than the 1000 mg/kg gavage dose administered four times a week by Belpoggi et al. (1995) who saw a carcinogenic response in Leydig cells; however, the greater frequency of dosing in our experiment (daily vs. 4× per week) resulted in slightly greater cumulative MTBE exposure in the highest dose group over an equivalent 28-day period in the Belpoggi study, when cumulative dose is defined as daily dose in mg/kg/day × number of days of dosing per week. Animals received 280, 2800, or 5600 mg/kg/week in our study and 1000 and 4000 mg/kg/week in the two-year study conducted by Belpoggi et al. Group mean body weights were 468–476 (p > 0.05) after random assignment to groups seven days before initiating treatment (at day –7), at which time baseline testosterone was measured in plasma from tail vein blood samples. Preexposure and interim (day 14) blood samples were obtained from the tail using restraint without anesthesia. Animals were acclimated to the restrainer for two weeks before the pre-exposure sampling period. Interim sampling on day 14 occurred before treatment that day. Trunk blood was collected for analysis of serum hormones at sacrifice 16 to 20 h after the final MTBE dose on day 28. Organ weights, organ/body weight, and organ/brain ratios were also evaluated for evidence of hormone-related effects.

**Experiment 3.** A study of hormonal effects using castrated rats was designed to see if high gavage doses of MTBE directly affect hypothalamic-pituitary function. Subtle changes following chemical exposure in intact animals can go undetected because of sampling time and the strong feedback mechanisms regulating the hypothalamic-pituitary-gonadal axis. Changes in LH secretion by this portion of the axis can be detected more readily in castrated rats with testosterone levels controlled by diffusion from Silastic capsules vs. 4 times a week by Belpoggi et al. (1995) which point the elimination half-life of MTBE is about 47 min. The 800 mg/kg dose of MTBE in corn oil or corn oil only for up to 28 days, and portions of liver tissue were processed for either spectrophotometric measurement of peroxisomal β-oxidation (Lazarow, 1981) or electron microscopy. Gemfibrozil was incorporated into rodent chow (0.2 g/100 g feed) that was given to a control group for 14 days to produce positive control reference material for the β-oxidation assays. Hepatic peroxisome isolation was as summarized in Biegel et al. (1992). Left liver lobe sections collected for electron microscopy were rinsed in phosphate buffered saline, placed in fixative, and refrigerated until analysis. Terminal blood from MTBE- and corn oil-treated rats was collected in vacuum tubes without any additives, allowed to clot, and centrifuged for 15 min at 1200 × g to collect serum that was transmitted to a clinical laboratory. A standard clinical chemistry panel focusing on indicators of liver function was done using a Vitros 700 automated analyzer (Johnson & Johnson) that had been validated for veterinary samples.

**Experiment 4.** Hepatic peroxisome proliferation was the focus of a separate in vivo experiment conducted specifically to determine whether MTBE causes this effect. This is important for a thorough analysis of possible mechanisms of MTBE-associated Leydig cell cancer because a number of structurally diverse chemicals that increase hepatic peroxisomal activity, including gemfibrozil, ammonium perfluorooctanoate, and trichlorehylene, also cause Leydig cell tumors in rats (Clegg et al., 1997; Liu et al., 1996). In addition to causing P450 induction, liver hypertrophy, and liver cancer (in female mice), MTBE increases lipid peroxidation in mouse liver microsomes (Katoh et al., 1993), all of which are characteristics of peroxisome proliferators. To determine whether MTBE might have such an effect in male rats, additional groups of Sprague-Dawley rats were gavaged with high doses of MTBE (800 mg/kg) in corn oil or corn oil only for up to 28 days, and portions of liver tissue were processed for either spectrophotometric measurement of peroxisomal β-oxidation (Lazarow, 1981) or electron microscopy. Gemfibrozil was incorporated into rodent chow (0.2 g/100 g feed) that was given to a control group for 14 days to produce positive control reference material for the β-oxidation assays. Hepatic peroxisome isolation was as summarized in Biegel et al. (1992). Left liver lobe sections collected for electron microscopy were rinsed in phosphate buffered saline, placed in fixative, and refrigerated until analysis. Terminal blood from MTBE- and corn oil-treated rats was collected in vacuum tubes without any additives, allowed to clot, and centrifuged for 15 min at 1200 × g to collect serum that was transmitted to a clinical laboratory. A standard clinical chemistry panel focusing on indicators of liver function was done using a Vitros 700 automated analyzer (Johnson & Johnson) that had been validated for veterinary samples.

**Experiment 5.** The relationships between estradiol, testosterone, and aromatase (P450arom) activity in liver and testis after high MTBE exposures were studied in this experiment intended to address several possible scenarios. Aromatase induction would increase conversion of testosterone to estradiol, which might explain lower testosterone levels that had been observed previously with MTBE. In addition, increased circulating estradiol, like increased testosterone levels, will decrease LH production and thereby indirectly reduce testosterone production by Leydig cells. Alternatively, aromatase inhibition, which is a known mechanism of some Leydig cell carcinogens, had never been investigated using MTBE. This could reduce circulating estradiol, thus eliminating some of the negative feedback on the anterior pituitary, resulting in increased LH which, if prolonged, could result in Leydig cell hyperplasia. Treatment was by gavage with 1200 mg MTBE/kg/day or corn oil vehicle for 14 consecutive days, a dosing regimen that we had determined in prior experiments to be able to lower testosterone without significant adverse effects on body weight, and which was also close to the 1000 mg/kg/day dose used in the 2 year gavage cancer bioassay (Belpoggi et al., 1995). Rats were sedated with CO2, and decapitated 1 h following the last dose of MTBE. Trunk blood for plasma hormones was collected into vacutainer tubes containing heparin. Liver, testes, accessory sex organs, and brain were removed and weighed. Plasma samples were stored at –70°C until analysis of estradiol and testosterone. Liver and testis microsomes were isolated by differential centrifugation. Total protein content was determined using the Coomassie Plus Assay (Pierce Chemical, Rockford, IL). Total cytochrome P450 was measured in liver microsomes by the method of Omura and Sato (1967).

Aromatase activity was measured using the tritiated water-release method of Lehart and Simpson (1991). Microsomes used to measure aromatase activity were diluted in pH 7.4 buffer (assay buffer) to a protein concentration of 1.5 mg/ml. A 100-μl aliquot of microsomes, 500 μl of assay buffer, and 370 μl of reaction buffer, consisting of 1.25 μl/m of [1β-1H] androstened-4-ene-3,17-dione (Amersham Pharmacia Biotech, Piscataway, NJ) prepared in assay buffer were added to each test tube. Assay blanks contained 100 ml of assay buffer instead of microsomal suspension. Reactions were initiated by addition of 30 μl of cofactor mix (1 mM NADPH, 10 mM glucose-6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase prepared in assay buffer). After incubation in a water bath at 37°C for 3 h, reactions were stopped by placing the reaction vessels on ice. Samples were then extracted with 2 ml of chloroform, gently shaken, and centrifuged at 2000 rpm. The aqueous phase was...
removed and cleared with 1 ml of 2.5% activated charcoal, vortexed for 40 s, and centrifuged for 10 min at 2000 rpm. The tritiated water released into the aqueous phase was measured by scintillation counter. All samples were assayed in duplicate.

**Gavage doses related by PBPK to carcinogenic doses by inhalation.** A physiologically based pharmacokinetic model for MTBE in Fischer 344 rats (Borghoff et al., 1996) was used to relate the high gavage doses in Experiments 1–4 to the inhalation exposures causing Leydig cell tumors, assuming no appreciable differences in MTBE pharmacokinetics between rat strains. The 1500 mg/kg doses we used initially in Experiment 1 are equivalent to 6-h inhalation exposures of 4969 ppm, and the 800 mg/kg administered daily in other experiments is equivalent to 2493 ppm (J. Brown, personal communication). Our gavage doses were thus also close to the 5000 ppm and 3000 ppm concentrations of MTBE used in the 24-month cancer bioassay in Fischer 344 rats in which significant increases in Leydig cell cancers were observed at these high levels (Bird et al., 1997).

**Radioimmunoassays (RIA).** Total testosterone was measured using $^{125}$I Coat-a-Count kits (Diagnostic Products Corporation [DPC], Los Angeles, CA). Rat-specific $^{125}$I Coat-a-Count kits were used to measure corticosterone. Rat specific double antibody RIA kits were used to measure LH and prolactin (Amersham, Arlington Heights, IL). Estradiol was measured in extracted plasma samples using the DPC kit tracer and primary antibody, but other steps in the RIA were modified and optimized to detect estradiol levels as low as 2.5 pg/ml. Plasma aliquots of 500 μl were extracted with 2.0 ml of ethyl acetate: hexane (3:2), the bottom layer was quick frozen, and the top layer was decanted into another extraction tube, placed in a 37°C water bath and concentrated to dryness under a stream of compressed air. The air-dried samples were reconstituted to 500 μl using phosphate buffered saline (PBS), pH 7.0, containing 0.1% gelatin and analyzed in duplicate (200 μl per assay, as specified in the DPC kit). Estradiol standards were also made using this PBS-gelatin buffer. The DPC $^{125}$I–estradiol tracer was added according to kit instructions, but 50 μl of primary antibody from the kit was used in lieu of the usual 100 μl. A dextran charcoal slurry was substituted for the DPC second antibody precipitating solution to remove unbound tracer. All samples from an experiment were assayed in a single run using materials bearing one lot number. The intra-assay variability of this modified estradiol kit method was 5%, while intra-assay variability of the standard testosterone, corticosterone, prolactin, and LH assay kits was 2–3%.

**Isolated Leydig cell culture experiments.** MTBE and t-butanol, a major metabolite, were added to Leydig cells in culture to study direct effects on testosterone synthesis. Leydig cells from adult Sprague-Dawley rats were isolated by collagenase dispersion (Collagenase type 3, Worthington Biochemical, Fort Worth, TX) and Percoll density gradient purification (Biegel et al., 1993). The cells were maintained in phenol red-free RPMI containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cell viability was assessed before and after treatment by Trypan blue exclusion and was at least 85%. Cells were seeded in 12-well culture plates (Corning, Corning, NY) at a density of 100,000 cells/ml of culture medium per well and incubated for 1 h at 37°C in a humidified atmosphere of 5% CO₂:95% air. Medium was replaced containing different concentrations of test compounds, and wells testing their effects on gonadotropin-stimulated steroidogenesis also contained a final concentration of 2 IU/ml hCG (Sigma-Aldrich, St. Louis, MO). Aminoglutethimide was used as a positive control. Supernatants from each well were collected after 3 h and stored at 4°C for no more than 48 h prior to analysis for testosterone. Concentrations of testosterone produced were measured in 50 μl of cell supernatant using a Coat-a-Count testosterone RIA kit (Diagnostic Products Corporation, Los Angeles, CA).

**Statistical analysis.** Statistical significance ($p < 0.05$) was determined for all measurements initially by analysis of variance (ANOVA) or t-test (two-group study designs). Unpaired Student’s t-tests with Bonferroni corrections were used for multiple comparisons among groups. Regression analysis was used to evaluate significance of dose-related trends in organ weights.

**RESULTS**

**Weight Gain Suppression and Other Signs of Toxicity**

Nearly all rats receiving MTBE in Experiment 1 were noticeably sedated and ataxic for up to 60 min after each dose during the first two weeks of treatment. One rat in the 1500 mg/kg group died on day 11. Another rat in the 1000 mg/kg group was euthanized early in the experiment after swelling around the neck was observed that could have been caused by a gavaging accident, although necropsy could not confirm this. Mean weight loss in the 1500 mg/kg group was 6% over the first two weeks, but several animals experienced much greater weight reduction, after which time MTBE doses were reduced to allow MTBE-treated animals to recover from an initial body weight decline. By the end of this exploratory experiment, untreated animals had gained an average of 31 g over starting body weight, vehicle controls had gained 18 g, net gain of animals exposed initially to 1000 mg/kg was 6 g, and the group exposed initially to 1500 mg/kg had just regained their starting body weight, corresponding to net body weight increases of 8, 3, 1, and 0%, respectively.

In the 28-day Experiment 2, no animals died during the dosing period even though daily administration of 800 mg/kg MTBE resulted in a greater weekly cumulative exposure than experienced by any treatment group in Experiment 1. Average body weights (Fig. 1) in both the 800 mg/kg/day and 400 mg/kg/day dose groups were statistically different from corn oil controls at the end of treatment ($p < 0.05$). Body weight in the 800 mg/kg group began to decline dramatically only after two weeks of treatment, and only toward the end of the experiment in the 400 mg/kg dose group.

In the 14-day Experiment 5 the dose selection and careful attention to handling and other procedures that might cause unnecessary stress resulted in no significant difference in final body weight between the 1200 mg/kg MTBE and corresponding corn oil control group. This was important because signif-
Subchronic Oral MTBE in Male Rats

Table 1: Serum Testosterone and Liver P450 (mean ± SEM) in Sprague-Dawley Rats Receiving MTBE by Gavage (Experiment 1)

<table>
<thead>
<tr>
<th>Group</th>
<th>Testosterone (ng/ml)</th>
<th>Liver weight</th>
<th>Total liver protein</th>
<th>Liver P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 (4–5 h)</td>
<td>Day 14</td>
<td>Day 28</td>
<td></td>
</tr>
<tr>
<td>Pooled controls</td>
<td>2.34 ± 0.15 (4)</td>
<td>1.86 ± 0.22 (23)</td>
<td>1.58 ± 0.22 (22)</td>
<td>15.19 ± 0.31 (24)</td>
</tr>
<tr>
<td>1000 mg/kg MTBE</td>
<td>1.34 ± 0.18 (4)</td>
<td>1.42 ± 0.30 (11)</td>
<td>1.89 ± 0.31 (11)</td>
<td>15.49 ± 0.32 (11)</td>
</tr>
<tr>
<td>1500 mg/kg MTBE</td>
<td>0.62 ± 0.09 (4)*</td>
<td>1.01 ± 0.17 (11)</td>
<td>1.71 ± 0.23 (10)</td>
<td>15.20 ± 0.28 (11)</td>
</tr>
</tbody>
</table>

Note. Pooled controls include untreated and corn oil vehicle controls. MTBE groups initially receiving 1500 mg/kg and 1000 mg/kg were reduced to 750 and 500 mg/kg, respectively, beginning on day 13 to allow treated animals to regain body weight lost during the first weeks of treatment. Total liver protein given in mg/g.

* Statistically different from pooled control, p < 0.05.

Reduced Testosterone Soon after Dosing

The main hypothesis of Experiment 1 was that MTBE induction of hepatic P450 in male rats, initially reported by Brady et al. (1990), could result in increased steroid catabolism that would be manifested by reduced circulating testosterone levels concomitant with increased P450. Preliminary data were also gathered to explore a possible role for corticosterone and a generalized stress response; hence, the 4-h and 14-day tail vein sampling of some animals and analysis of corticosterone when remaining plasma volume allowed. Liver weights were no different at the end of this 28-day experiment (Table 1), and total hepatic P450 was increased, although only slightly even in the high dose group (p < 0.05, one-tailed t-test).

Contrary to expectation, in this experiment the P450 increase was not accompanied by a measurable change in circulating testosterone in the blood sampled on day 28 when livers were collected for P450 measurements. There was, however, a dramatic dose-related decrease in mean serum testosterone within just 4–5 h after the first dose, when levels after 1500 or 1000 mg/kg MTBE were 27 and 57% of control, respectively, with no difference between dose groups evident in this small sample size. At the day 14 sampling time there was also no effect of treatment, with control, low, and high dose groups averaging 373 ± 59, 378 ± 58, and 383 ± 53 ng/ml, respectively (n = 3, 4).

Concerned that the statistical reduction in testosterone observed at 4–5 h could have been biased by small group sizes, we then conducted an abbreviated follow-up experiment to confirm the testosterone depression soon after single, high doses of MTBE using group sizes of six rats. Preexposure baseline testosterone values were compared with postexposure values in each rat. Although the corn oil control group in this experiment had a higher preexposure baseline mean testosterone than the two MTBE treatment group means, 4 h after either the 1000 mg/kg or 1500 mg/kg gavage treatments the postexposure means (± SEM) were 47 ± 59% and 31 ± 34% of baseline in these groups, as compared with the corn oil controls, which were still 93 ± 33% of their preexposure baseline. Again, the treatment group mean corticosterone values did not differ. From this we concluded that high gavage doses of MTBE can be expected to reduce circulating testosterone in rats during the hours immediately following treatment.

Only Mild Effects on Hormone-Dependent Organ Weights

In Experiment 1, no significant differences were seen in absolute weights of liver, kidney, testis, seminal vesicles or epididymides or in organ:body weight ratios (data not shown). In the experiments that followed involving more frequent (daily) dosing, some subtle changes were observed. Table 2 showing absolute organ weights shows an increase in mean pituitary weight in the 400 mg/kg group relative to controls (p < 0.05). This difference was attributed to one pituitary that was over twice the size of any other recorded for this experiment. As in Experiment 1, both absolute liver weight and relative mean liver:body weight ratios were similar in all
groups at the end of 28 days of dosing. Testis weights were comparable in all groups, whereas androgen-dependent accessory sex organ weights appear to decrease slightly, and adrenal and thyroid glands tend to increase as the dose of MTBE increases, although no group is statistically different from control.

Brain weight was the same in all groups and is considered a more stable index for comparison of organ weights than organ:body weight (Table 3). The mild, dose-related reduction would be consistent with discontinuous support by circulating testosterone, which, even if reduced only intermittently, presumably occurred with each daily dose during this experiment. Dose-dependent adrenal:body weight (p < 0.01) and thyroid:body and thyroid:brain weight (p < 0.05) ratio increases are statistically significant.

**Corticosterone Increases and Little Consistent Change in Pituitary Hormone Levels**

Table 4 shows serum testosterone, corticosterone, LH, and prolactin after daily gavaging with MTBE for 28 days in Experiment 2. In all cases blood had been sampled over 12 h after the final MTBE treatment. Baseline testosterone levels were similar in all groups one week before beginning treatment (day –7), and on day 14 testosterone levels were still similar in all groups. At 28 days, individual testosterone levels of all but one of the animals in the 800 mg/kg dose group were noticeably lower than the control mean and the group average was 65% of corn oil controls (p > 0.05). One animal in the 800 mg/kg MTBE group had a high testosterone level of 3.08 ng/ml compared to all other animals in this group, which ranged from 0.25 to 1.70 ng/ml. The vehicle control range was 0.42–3.53 ng/ml.

Unlike in the prior experiments, corticosterone in both the 400 and 800 mg/kg treatment groups (p < 0.01) was higher than in the control group at 14 days (p < 0.01) and elevated again in the 800 mg/kg group at 28 days (p < 0.01). The difference in response from Experiment 1 (and its follow up) might be explained by small sample size, time of sampling (16–20 h versus 4–5 h) after gavaging the animals, inability to detect a dose-response when all doses are extremely high, or a more complicated scenario involving multiple interactions in various organs. Biphasic responses are seen in other studies of the combined effects of glucocorticoids and steroids at receptors in the hypothalamic-pituitary-gonadal and hypotha-

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final bw</th>
<th>Liver</th>
<th>Pituitary</th>
<th>Testes</th>
<th>Epididymides</th>
<th>Prostate</th>
<th>SV Accessory organs</th>
<th>Adrenals</th>
<th>Thyroid</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>3.05 ± 0.08</td>
<td>1.09 ± 0.02</td>
<td>0.48 ± 0.03</td>
<td>1.46 ± 0.10</td>
<td>3.03 ± 0.13</td>
<td>45.0 ± 0.13</td>
<td>18 ± 0.0</td>
<td>18 ± 0.0</td>
<td>2.05 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>MTBE (mg/kg/day)</td>
<td>40</td>
<td>517 ± 22</td>
<td>15.0 ± 1.1</td>
<td>11 ± 0.0</td>
<td>2.91 ± 0.09</td>
<td>1.08 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>1.31 ± 0.09</td>
<td>2.85 ± 0.13</td>
<td>47 ± 0.0</td>
</tr>
<tr>
<td>400</td>
<td>487 ± 19</td>
<td>14.0 ± 0.7</td>
<td>13 ± 0.0*</td>
<td>3.10 ± 0.05</td>
<td>1.05 ± 0.02</td>
<td>0.45 ± 0.05</td>
<td>1.33 ± 0.08</td>
<td>2.85 ± 0.12</td>
<td>50 ± 0.0</td>
<td>19 ± 0.0</td>
</tr>
<tr>
<td>800</td>
<td>459 ± 16*</td>
<td>13.9 ± 0.6</td>
<td>11 ± 0.0</td>
<td>2.82 ± 0.20</td>
<td>1.05 ± 0.08</td>
<td>0.43 ± 0.04</td>
<td>1.17 ± 0.07</td>
<td>2.64 ± 0.13</td>
<td>49 ± 0.0</td>
<td>23 ± 0.0</td>
</tr>
</tbody>
</table>

Note. Body weight (bw) and organ weights are given in g except for pituitary, adrenals, and thyroid, given in mg; n = 12–13. Combined accessory organs, epididymides + prostate + SV (seminal vesicles).
* Significantly different from corn oil control, p < 0.05.

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Accessory organs</th>
<th>Adrenals</th>
<th>Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SEM, Experiment 2)</td>
<td>(Mean ± SEM, Experiment 2)</td>
<td>(Mean ± SEM, Experiment 2)</td>
<td>(Mean ± SEM, Experiment 2)</td>
</tr>
<tr>
<td>Corn oil</td>
<td>2.92 ± 0.01</td>
<td>0.58 ± 0.03</td>
<td>0.86 ± 0.02</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>MTBE (mg/kg/day)</td>
<td>40</td>
<td>3.30 ± 0.30</td>
<td>0.55 ± 0.02</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>400</td>
<td>2.95 ± 0.22</td>
<td>0.59 ± 0.03</td>
<td>1.04 ± 0.08</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>800</td>
<td>2.94 ± 0.16</td>
<td>0.58 ± 0.03</td>
<td>1.08 ± 0.08</td>
<td>0.48 ± 0.07</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; significant trends by regression analysis.
lamic-pituitary-adrenal axes, depending on other hormone levels present at that time.

LH and prolactin were similar in all groups at 28 days (p > 0.05). A few day 14 tail vein samples contained sufficient serum volume to allow measurement of LH in this experiment. Mean serum LH values were 0.14 ± 0.07, 0.36 ± 0.31, and 0.59 ± 0.20 in the control, 40, and 400 mg/kg groups, respectively (n = 3, 4, and 3), indicating a possible dose-related increase. Only two values were available for remaining samples from the 800 mg/kg group (0.58 ng/ml and 1.25 ng/ml). When all animals in this experiment were later sampled at 28 days 16–20 h after the final dose, there were no differences in LH between controls and animals treated with up to 800 mg/kg.

**Estradiol, T, LH, and Aromatase Changes in a 14-Day Experiment**

In Experiment 5 (Table 5), rats were sacrificed and blood was collected on day 14 one hour after the final daily dose of 1200 mg/kg MTBE or corn oil. As predicted from prior experiments, plasma testosterone in the MTBE-treated group was decreased to 49% of control (p < 0.05). No changes in absolute or relative accessory sex organ weights were apparent at this time, but mean LH was 90% of the control mean while estradiol was significantly increased to 136% of control (both p < 0.05). Both hepatic and testicular aromatase activity were significantly reduced to 64% and 45% of control (p < 0.05), respectively. Liver weight in the treated group was noticeably greater (p < 0.05); however, total hepatic P450 in the MTBE-treated group was no different from control.

**No Evidence of Direct Effect of Short-Term Exposure on Gonadotropin Production/Secretion in a Castrate Rat Model**

Two similar trials in Experiment 3 focused on other hypothalamic-pituitary-gonadal axis organs other than the testis as possible primary targets. Plasma samples collected at 4 h on day 1 and on day 5 of MTBE treatment (Table 6) coincide with three and eight days after castration. As expected using this model, circulating testosterone was higher and LH was lower in animals implanted with Silastic tubing containing testosterone propionate compared with sham implanted controls. Animals were matched by body weight initially and all implants contained a standard amount of testosterone; however, group weights were no longer well matched after recovery from

**TABLE 4**

Plasma Hormone Concentrations in Male Sprague-Dawley Rats Treated Daily with MTBE by Gavage for 28 Days (Experiment 2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Day –7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 28</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBE (mg/kg/day)</td>
<td>11–12</td>
<td>1.53 ± 0.28</td>
<td>1.96 ± 0.35</td>
<td>1.72 ± 0.33</td>
<td>290 ± 33</td>
<td>239 ± 19</td>
<td>1.24 ± 0.34</td>
<td>11.04 ± 3.06</td>
</tr>
<tr>
<td>40</td>
<td>10–13</td>
<td>1.57 ± 0.31</td>
<td>1.96 ± 0.27</td>
<td>2.13 ± 0.44</td>
<td>405 ± 55**</td>
<td>274 ± 32</td>
<td>1.12 ± 0.33</td>
<td>9.56 ± 1.84</td>
</tr>
<tr>
<td>400</td>
<td>10–13</td>
<td>1.54 ± 0.21</td>
<td>2.58 ± 0.41</td>
<td>1.23 ± 0.29</td>
<td>504 ± 45**</td>
<td>244 ± 42</td>
<td>1.15 ± 0.29</td>
<td>9.22 ± 1.53</td>
</tr>
<tr>
<td>800</td>
<td>10–13</td>
<td>1.53 ± 0.24</td>
<td>1.98 ± 0.25</td>
<td>1.12 ± 0.21*</td>
<td>505 ± 16**</td>
<td>429 ± 49*</td>
<td>1.27 ± 0.37</td>
<td>11.2 ± 2.19</td>
</tr>
</tbody>
</table>

*Note. Values (ng/ml) are hormone concentrations on experiment day sampled (mean ± SEM). *p < 0.05, **p < 0.01; statistically significant differences from corn oil vehicle controls.

**TABLE 5**

Plasma Hormones and Tissue Aromatase Activity in 14 Day Gavage Experiment in Male Sprague-Dawley Rats (mean ± SEM, Experiment 5)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight</th>
<th>Plasma</th>
<th>Aromatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Liver:bw</td>
</tr>
<tr>
<td>Corn oil control</td>
<td>386 ± 7</td>
<td>396 ± 7</td>
<td>0.034 ± 0.001</td>
</tr>
<tr>
<td>MTBE</td>
<td>1200 mg/kg</td>
<td>388 ± 6</td>
<td>393 ± 5</td>
</tr>
</tbody>
</table>

*Note. All values shown in table are mean ± SEM, n = 10. Liver P450 is given in nm P450/mg total protein; liver aromatase is given in pmol/h/mg microsomal protein; testicular aromatase is given in pmol/h/mg microsomal protein × 10⁻². Testosterone is given in ng/ml; estradiol is given in pg/ml; LH (luteinizing hormone) is given in mg/ml.

* p < 0.05, ** p < 0.01; statistically significant differences from corn oil vehicle control.
surgery when the treatments began, so body weight was included in the ANOVA as a covariate.

Comparing hormone levels in the sham implant animals treated with either corn oil or MTBE (Table 6), differences in group mean testosterone were not statistically different at either 4 h or day 5 time points in either experiment. LH levels continued to rise similarly in both MTBE and corn oil treated groups over the five days as testosterone gradually declined after castration, indicating that any modulating effect of MTBE on the hypothalamus or pituitary could not be detected under these conditions. In Trial 1, mean LH appears to be slightly higher in the group of sham implant rats treated for five days with MTBE compared to the corresponding sham-implanted corn oil control (Table 6, far right column). No such difference was seen in these groups in Trial 2. In Trial 1 groups supplemented with testosterone propionate (TP), both 4 h and day 5 LH in control animals appear to be higher than in the MTBE-treated group; however, this difference can be explained by one potentially confounding control animal that gained considerably more weight than expected while receiving the same amount of testosterone as the other animals. This caused this animal to have the lowest blood levels of testosterone and consequently higher LH values. These apparent differences in means are not significant when weight is considered as a covariate, and any apparent effect observed in Trial 1 was not replicated in Trial 2.

Measuring selected hormone-dependent organ weights in this study also shed some light on whether MTBE might enhance or diminish tissue responsiveness to testosterone, which could manifest itself as different pituitary or accessory organ weights in MTBE-treated groups relative to corn oil controls. Overall, there was no clear indication from pituitary or accessory organ weights that these organs had been affected directly by MTBE under the conditions of this experiment (not shown).

**Suppressed Testosterone Production by Isolated Leydig Cells**

Significant inhibition of both basal and hCG-stimulated testosterone production was observed in isolated rat Leydig cells treated with either 50 mM or 100 mM concentrations of MTBE or t-butanol (Fig. 2). 50 mM t-butanol reduced hCG-stimulated testosterone production to 27% of the RPMI vehicle control while 50 mM MTBE caused a 50% reduction. Neither MTBE nor its major metabolite t-butanol was as potent as 5 mM aminoglutethimide. The following assumptions were made to relate 100 mM (8815 g/ml) and 50 mM (4408 g/ml) MTBE to reasonably expected in vivo tissue concentrations following inhalation treatments similar to those used in the first cancer bioassay in which Leydig cell tumors were observed (Bird et al., 1997). Referring to Figure 2 in the pharmacokinetic analysis reported by Miller et al. (1997), it appears that a 6 h, 8000 ppm inhalation exposure results in a peak plasma MTBE concentration of approximately 560 g/ml. Borghoff et al. (1996) estimated organ:blood partition coefficients for Fischer 344 rats ranging from 0.56:1 (muscle) to 3.11:1 (liver). Partitioning in the rat testis may be expected to fall somewhere within this range, based on measurements of lipid:organ weight percentages in organs of normal untreated male rats (Kee and Smith, 1996). This would suggest that the rat testis might
No Evidence of Peroxisome Proliferation (Experiment 4)

Mean (± SD) liver:body weight ratios of corn oil control, MTBE (1000 mg/kg), and gemfibrozil groups were 3.40 ± 0.51%, 3.77 ± 0.21%, and 5.32 ± 0.53% g, respectively. In addition to significantly increased liver weight in the positive control group, gemfibrozil produced nearly a 16-fold increase over controls in β-oxidation activity, but MTBE did not increase β-oxidation. Although MTBE did not markedly increase liver mass or increase β-oxidation activity relative to controls after 14 days of gavaging with 1000 mg/kg in two separate trials, some evidence of liver toxicity was manifested in higher AST or ALT values in at least four of the six treated rats sampled relative to controls. Mean serum cholesterol levels for MTBE and control groups were 68.8 ± 5.0 mg/dl and 69.3 ± 11.0 mg/dl, respectively. Triglyceride levels were also unchanged: 53.5 ± 5.0 mg/dl in MTBE-treated animals and 49.3 ± 10.8 mg/dl in control animals. Electron microscopic observation of liver sections from MTBE-treated rats also showed no evidence of peroxisome proliferation (not shown, J. K. Reddy, personal communication) consistent with the lack of activity in the β-oxidation assay at doses causing elevated AST or ALT.

DISCUSSION

Relating effects of MTBE to hormonal patterns and other effects of rat Leydig cell carcinogens with known mechanisms (Table 7) revealed several ways in which MTBE could have increased the incidence of these tumors after high exposures used in cancer bioassays. Decreased testosterone is expected with several of these mechanisms, but each has other characteristics that provided additional mechanistic clues.

Direct effects on Leydig cell steroidogenesis were supported by in vitro experiments and three separate in vivo experiments showing reduced testosterone at times when there was no clear evidence that reduced LH, decreased prolactin, or increased corticosterone were primarily responsible for the decrease (Experiments 1, 2, and 5). Different doses and elapsed times between treatment and hormone sampling, coupled with the relatively short half-life and rapid elimination of MTBE and t-butanol, could explain most of the in vivo testosterone observations reported by us and Williams et al. (2000). Decreases we observed were significant when measured within 1–5 h after dosing with 1000–1500 mg/kg (Experiments 1 and 5), but not when measured 16–20 h after the last of 28 daily doses with 800 mg/kg (Experiment 2). Williams et al. (2000) reported reduced testosterone in interstitial fluid 1 h after daily dosing with 1500 mg/kg on day 15 (in the absence of a change in LH), although not in 28-day treated rats that were also sampled 1 h after dosing. This last observation is not consistent with the concept of direct transient steroidogenesis enzyme inhibition following MTBE exposure and rapid clearance, but changes in circulating testosterone caused by weak testosterone biosynthesis inhibitors can be missed in vivo (Fort et al., 1995). Also, LH levels may remain within the normal range (Clegg et al., 1997) or restore hormonal homeostasis by the time sampling occurs.

Increased LH production is the normal compensatory response of the pituitary to reduced circulating testosterone. Prolonged hyperstimulation of Leydig cells to restore testosterone levels is believed to be involved in several mechanisms of rat Leydig cell hyperplasia and neoplasia (Cook et al., 1999). LH levels in intact rats exposed to MTBE have been inconsistent. Sampling in Experiment 1 (40–800 mg/kg/day) revealed no change on day 28, a possible increase on day 14 in this experiment, and an increase on day 14 in Experiment 5 (1200 mg/kg/day) 1 h after dosing. In contrast, Williams et al. (2000) reported a dose-related decrease in LH 1 h after 28 days of treatment with 1500 mg/kg but little or no change after 15
days of treatment. Normally high LH variability could have masked subtle or transient effects. In experiments using castrate rats, although group sizes were small, we detected no direct effect of MTBE on pituitary gonadotropins, which prompted us to focus more on the testis as a site of action.

Estradiol and aromatase activity had not been measured previously in male rats treated with MTBE. Increased circulating levels of estradiol observed in Experiment 5 along with reduced testosterone suggest increased aromatase activity; however, we found reduced activity of aromatase in liver and testis microsomes from rats gavaged daily for 14 days with 1200 mg/kg of MTBE, and this combination of effects remains to be fully explained. Elevated estradiol can cause competitive product inhibition (Shimizu et al., 1993), which would then decrease aromatase activity, but what caused the estradiol increase was not clear. Aromatase inhibitors have caused Leydig cell tumors in dogs, presumably by reducing negative feedback by estradiol on the hypothalamic-pituitary-testicular axis, which leads to chronically increased LH. Estradiol itself is a mouse Leydig cell carcinogen, although it is not known to have this effect in rats (Clegg et al., 1997; Cook et al., 1999).

Two-week gavage studies of the rat Leydig cell carcinogen ammonium perfluorooctanoate in male rats showed a similar hormonal pattern to MTBE (Cook et al., 1992), and although aromatase induction was suggested it was not actually measured. In any case, the pattern of hormonal and aromatase changes observed in our study suggest greater complexity than aromatase inhibition alone as an explanation for rat Leydig cell tumors in MTBE cancer bioassays.

Williams et al. (2000) observed significantly decreased circulating dihydrotestosterone (DHT) in rats with testosterone levels similar to controls after 28 days of dosing with 1500 mg/kg MTBE. Finasteride, a 5α-reductase inhibitor, suppresses conversion of testosterone to the more potent DHT and reduces prostate and seminal vesicle weights and increases circulating LH, but does not markedly alter circulating testosterone in rats (O’Connor et al., 1998). The minimal effect of MTBE on accessory sex organ weights and LH suggest that 5α-reductase inhibition is not a major mode of action.

Other known mechanisms of rat Leydig cell carcinogenesis in Table 7 were ultimately not supported by our measurements.

---

**TABLE 7**

<table>
<thead>
<tr>
<th>Mechanisms and prototypes</th>
<th>Distinguishing characteristics of prototypes</th>
<th>MTBE effects (reported in this article and Williams et al., 2000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone biosynthesis inhibition (lansoprazole)</td>
<td>↓ circulating T  ↓ T production in vitro</td>
<td>↓ circulating T  ↓ T production in vitro</td>
</tr>
<tr>
<td>Fort et al., 1995</td>
<td>LH: ↑ or no detectable change</td>
<td>LH: results vary depending on dose and sampling time</td>
</tr>
<tr>
<td>Androgen receptor antagonism (Flutamide, linuron, procymidone)</td>
<td>↑ Accessory sex organ weights</td>
<td>Accessory organ weight reductions not statistically significant after one month of daily oral dosing</td>
</tr>
<tr>
<td>Cook et al., 1993; Murakami et al., 1995; Neuman, 1991</td>
<td>↑ T</td>
<td>LH: results vary depending on dose and sampling time</td>
</tr>
<tr>
<td>Aromatase inhibition</td>
<td>Decreased aromatase activity</td>
<td>Decreased aromatase activity in liver and testis microsomes</td>
</tr>
<tr>
<td>Clegg et al., 1997; Cook et al., 1999</td>
<td>↑ LH, ↓ estradiol</td>
<td>LH: results vary depending on dose and sampling time</td>
</tr>
</tbody>
</table>
Androgen receptor antagonists block negative feedback at pituitary and hypothalamic testosterone receptors, thereby stimulating LH production, but also tend to elevate circulating testosterone and cause marked atrophy of accessory organs (Cook et al., 1993; Murakami et al., 1995; Neumann, 1991). None of these characteristic effects was observed, so we concluded that MTBE is not likely to be an androgen antagonist. The dopamine agonist mesulergine decreases prolactin, thereby decreasing rat Leydig cell gonadotropin receptor number and testosterone synthesis (Prentice et al., 1992), which leads to increased LH stimulation of Leydig cells to restore normal testosterone levels. While we observed no effects on circulating prolactin in intact rats (Experiment 2) or gonadectomized rats (Experiment 3), Williams et al. (2000) reported decreased circulating prolactin in rats treated for 15 days but not 28 days. Inconsistent reports on the effect of MTBE with the expected hormonal pattern of dopamine agonism tend to rule out that mechanism as the cause of Leydig cell tumors in MTBE cancer bioassays. We also saw no evidence of peroxisome proliferation using biochemical and electron microscopic methods.

MTBE increases hepatic metabolism of estrogen in female mice (Moser et al., 1996), which may have led to increased liver tumors seen in female mice (Bird et al., 1997). We and others (Williams and Borghoff, 2000) initially hypothesized an analogous mechanism of Leydig cell tumorigenesis that could have resulted from persistently high LH levels caused indirectly by increased catabolism of testosterone, recognizing that no Leydig cell carcinogens are known yet to act through this mechanism (Cook et al., 1999). Williams and Borghoff (2000) subsequently concluded that the magnitude of induction caused by high doses of MTBE is unlikely to explain decreases in serum testosterone by enhanced hepatic clearance. Total hepatic P450 and serum testosterone measured in the same rats at the end of our first 28 day experiment using high doses of MTBE (Experiment 1) showed that while P450 was increased (1.6-fold control), there was no detectable reduction in testosterone at that time. In Experiment 4, total P450 was not markedly changed at a time (day 14) when testosterone was reduced by 50%. Maintenance of steroid levels is under complex control, so we cannot rule out altered hepatic metabolism as a possible contributor to the endocrine changes seen with MTBE; however, evidence increasingly points to other more direct effects on the hypothalamic-pituitary-gonadal axis.

The adrenal cortex is more vulnerable to chemically induced injury than any other endocrine tissue (Ribelin, 1984). Repeated ether stress increases circulating ACTH and adrenal secretion of corticosterone, and decreases plasma concentrations of LH and testosterone, while the pituitary response to LH-releasing hormone (LH-RH) and testicular response to LH are both maintained (Tohei et al., 1997). Corticosterone was not consistently elevated in MTBE-treated rats with reduced testosterone, so it is not clear how this “stress” hormone may have contributed to the effects observed. The dose-related increase in both thyroid:brain and thyroid:body weight ratios after 28 days of dosing with MTBE (Experiment 2) appears to be the first report of this effect and could be a compensatory response to reduced weight gain also seen in this experiment. Alternatively, Williams et al. (2000) observed reduced serum T<sub>3</sub> in rats after one month of treatment with 1000 and 1500 mg/kg/day by gavage. Enlarged thyroid glands might be expected if low circulating T<sub>3</sub> persists long enough to cause a compensatory, sustained increase in pituitary TSH secretion. Thyroid hormones regulate Leydig cell steroidogenesis (Papadopoulos, 1999) and expression of liver P450 enzymes involved in steroid catabolism (Waxman and Chang, 1995). Further studies are needed to understand how high doses of MTBE affect thyroid function and how this might alter Leydig cell tumor incidence and other effects observed.

Rat Leydig cell carcinogens may act through multiple mechanisms, and their effects can be compounded by stress and normal age-related changes that cause a high background incidence in many strains (Cook et al., 1999). While the subchronic dosing and in vitro experiments described here do not fully explain the occurrence of Leydig cell tumors 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 mechanism. They also identified changes in the liver, thyroid, and adrenals as plausible contributors to this effect that warrant further consideration in future studies for a more complete understanding of MTBE’s carcinogenic potential.

ACKNOWLEDGMENTS

Human chorionic gonadotropin was a gift from WIL Research Laboratories, Inc. Oral-inhalation dose equivalency information using the MTBE PBPK model for F-344 rats (Borghoff et al., 1996) was provided by J. Brown, California Environmental Protection Agency. We thank J. Reddy for electron microscopy analysis of peroxisome proliferation in liver samples from MTBE-treated animals; D. Colarusso of Biomedical Testing Service, San Diego, for the standard clinical chemistry analysis; V. Lance, Department of Endocrinology, San Diego Zoo, for assistance with optimizing estradiol measurements; A. Bakarich, W. Hayhow, and H. Gonzales for other valuable technical assistance; and R. Chapin for helpful comments on an early draft of this manuscript. Contributions from the Oxygenated Fuels Association, a faculty Grant-in-Aid from San Diego State University, and student grants-in-aid from Sigma Xi, the Air and Waste Management Association, and ARCO/Lyondell Chemical Company helped to support this research.

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