Two-Year Toxicity and Carcinogenicity Study of Methyleugenol in F344/N Rats and B6C3F$_1$ Mice

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Methyleugenol (MEG) was tested for toxicity/carcinogenicity in a 2-yr carcinogenesis bioassay because of its widespread use in a variety of foods, beverages, and cosmetics as well as its structural resemblance to the known carcinogen safrole. F344/N rats and B6C3F$_1$ mice (50 animals/sex/dose group) were given MEG suspended in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg/day for 2 yr. Control groups (60 rats/sex and 50 mice/sex) received only the vehicle. A stop-exposure group of 60 rats/sex received 300 mg/kg/day by gavage for 53 weeks followed by the vehicle only for the remaining 52 weeks of the study. A special study group (10 animals/sex/species/dose group) were used for toxicokinetic studies. All male rats given 150 and 300 mg/kg/day died before the end of the study; survival of female rats given 150 mg/kg/day and all treated female mice was decreased. Mean body weights of treated male and female rats and mice were decreased when compared to control. Area under the curve results indicated that greater than dose proportional increases in plasma MEG occurred for male 150 and 300 mg/kg/day group rats (6 and 12 month) and male 150 mg/kg/day mice (12 month). Target organs included the liver, glandular stomach, forestomach (female rats) and kidney, mammary gland, and subcutaneous tissue (male rats). Liver neoplasms occurred in all dose groups of rats and mice and included hepatoadenoma, hepatocarcinoma, hepatocholangioma (rats only), hepatocholangiocarcinoma, and hepatoblastoma (mice only). Nonneoplastic liver lesions included eosinophilic and mixed cell foci (rats only), hypertrophy, oval cell hyperplasia, cystic degeneration (rats only), and bile duct hyperplasia. Mice also exhibited necrosis, hematopoietic cell proliferation, and hemosiderin pigmentation. Glandular stomach lesions in rats and mice included benign and malignant neuroendocrine tumors, neuroendocrine cell hyperplasia, and atrophy and in mice included glandular ectasia/chronic active inflammation. In female rats, the forestomach showed a positive trend in the incidences of squamous cell papilloma or carcinoma (combined). Male rats also exhibited kidney (renal tubule hyperplasia, nephropathy, and adenomacarcinoma), mammary gland (fibroadenoma), and subcutaneous tissue (fibroma, fibrosarcoma) lesions. Male rats also exhibited malignant mesotheliomas and splenic fibrosis. These data demonstrate that MEG is a multisite, multispecies carcinogen.

Keywords: *Methyleugenol; toxicity; carcinogenicity; toxicokinetics; rats; mice*

INTRODUCTION

Methyleugenol (MEG) has a high potential for human exposure, which occurs primarily through the consumption of foods and beverages. MEG concentrations in these products range from 4.8 to 52 ppm (Furia and Bellanca, 1975). MEG is a natural constituent of various essential oils, e.g., citronella, anise, nutmeg, cinnamon (FEMA, 1978; Furia and Bellanca, 1975; Carlini et al., 1981; *Farm Chemical Handbook*, 1992), as well as fruits and vegetables, e.g., bananas and black peppers (WHO, 1981; MacGregor et al., 1974). The per capita intake of MEG is estimated to be 0.073 mg/day (WHO, 1981) or, based on more recent determinations and expressed as a function of body weight, 0.26 μg/kg/day (Stofberg and Grundschober, 1987; NAS, 1989). Human exposure to MEG also occurs as a result of its use as a fragrance in perfumes, creams, lotions, soaps, and detergents (Opdyke, 1979). The estimated human average consumption is approximately 6 μg of MEG/day (Miller et al., 1983). Occupational exposure to MEG involves approximately 3000 workers annually (NIOSH, 1990).

MEG is classified as an alkenylbenzene compound, and some of the chemicals in this class are known rodent carcinogens. For example, safrole, which is structurally similar to MEG, has been reported to induce hepatic, pulmonary, and renal tumors in mice and rats (IARC, 1976; Vesselinovitch et al., 1979; Miller et al., 1983). Estragole, which only differs from MEG in that it has one rather than two methoxy benzene-ring substituents, produced hepatomas in mice (Miller et al., 1983; CCRIS, 1998). Eugenol, the closest related alkenylbenzene to MEG, was tested for carcinogenicity in B6C3F$_1$ mice, but the only equivocal evidence was obtained regarding an increased incidence of liver tumors (NTP, 1983). The carcinogenic activity of these compounds led to further testing of other alkenylbenzene compounds, including MEG. B6C3F$_1$ mice were given intraperitoneal injections of MEG (total dose 4.75 μg) once a week for 4 weeks prior to weaning and were then examined at 13

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and up to 18 months (Miller et al., 1983). At 13 months, 70% of the treated mice had hepatomas as compared to 5% in the control group, and after 18 months, 96% of the MEG-treated mice had hepatomas as compared to 41% in the control group.

Standard genotoxicity studies with MEG were largely negative using the *Salmonella typhimurium* gene mutation assay (Sekizawa and Shibamoto, 1982; Mortelmans et al., 1986; Kettering and Torabinejad, 1995) and the *Escherichia coli* WP2 uvrAgene reversion test (Sekizawa and Shibamoto, 1982). However, positive, dose-related responses were observed with MEG in chromosomal recombination studies (Schiestl et al., 1989; Brennan et al., 1996), unscheduled DNA synthesis assays (Howes et al., 1990; Chan and Caldwell, 1992; Gardner et al., 1997), and DNA adduct formation tests (Gardner et al., 1997; Phillips et al., 1984), indicating more extensive metabolic activation is needed for MEG to express genotoxicity than is present in bacterial assay systems.

The carcinogenic activity of alkenylbenzene compounds requires metabolic activation. Safrole and estragole are metabolized by the liver, and their metabolites are more active hepatocarcinogens than the parent compounds (Borchert et al., 1973a,b; Drinkwater et al., 1976; Swanson et al., 1981). Alkenylbenzene compounds are metabolized by liver cytochrome P450 enzymes (Gardner et al., 1997), which results in the formation of epoxide and 1′-hydroxy metabolites (Wislocki et al., 1976; Delaforge et al., 1980a). While epoxides, in general, have been shown to be highly reactive with cellular components (Sims and Grover, 1974), the alkenylbenzene epoxide molecule is relatively stable (Delaforge et al., 1980a,b). In contrast, the 1′-hydroxy metabolite undergoes sulfate conjugation by hepatic sulfotransferase. As the sulfate conjugate, the O-sulfate is easily hydrolyzed, thereby leaving a carbonium ion intermediate (Miller and Miller, 1977) that reacts with DNA (Chan and Caldwell, 1992) and is believed to be the ultimate carcinogen for alkenylbenzene compounds (Miller et al., 1983). The 1′-hydroxy metabolite of estragole has been shown to react with DNA bases guanine and adenine (Phillips et al., 1984; Wiseman et al., 1987). ^{32}P-Postlabeling assays demonstrated that safrole, estragole, and MEG bind to DNA and have a high affinity for DNA (Phillips et al., 1984; Randerath et al., 1984).

On the basis of the high potential for human exposure and structural similarity to known carcinogens, MEG was nominated by the National Cancer Institute and Food & Drug Administration (FDA) for toxicity and carcinogenicity testing by the National Toxicology Program (NTP). The study reported here describes the in-life and pathology results of the 2-yr chronic gavage toxicity/carcinogenicity study of MEG in F344 rats and B6C3F$_1$ mice.

MATERIALS AND METHODS

Materials. Methyleugenol (1-allyl-3,4-dimethoxybenzene; CAS Registry No. 93-15-2; Lot 9224705) was obtained from Elan Chemical Company (Newark, NJ). The identity of the test article was verified by IR and NMR spectroscopy. Purity was ~99% as determined by HPLC using Ultracarb ODS (30) column with a detection wavelength of 280 nm, a flow rate of 0.7 mL/min, and an isocratic solvent system of 40% water: 60% acetonitrile. MEG was stored at room temperature in amber glass bottles with Teflon-lined lids. Methylcellulose (USP/FCC grade; Lots 876672 and 946150) was obtained from Fisher Scientific Company (St. Louis, MO). The identity was verified using IR, UV/visible, and NMR spectroscopy. Purity was determined by elemental analyses (0.06% sodium; experimental agreed with theoretical carbon/hydrogen molecule count), Karl Fischer water analysis (1.94% water), functional group titration (30.62% methoxy group content), HPLC (one major peak and no impurities with areas of 0.1% or greater relative to major peak area), and the complete battery of United States Pharmacopeia (USP) XXI analyses.

Animals. Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY). Upon arrival, animals were quarantined for 11−12 day. At randomization (day −4), the approximate ages and weight ranges were 5−6 weeks and 94−130 and 82−106 g for the male and female rats, respectively, and 6−7 weeks and 21−26 and 17−21 g for the male and female mice, respectively. Animals were housed in polycarbonate cages [3 (males) or 5 (females)/cage for rats; individually (males) or 5 (females)/cage for mice] that contained bedding material (Sani-Chips, P. J. Murphy Forest Products Corp., Montville, NJ). Environmental controls for animals rooms were set to maintain temperature at 69−75 °C, 35−65% relative humidity, 10 fresh air changes/h, 12 h light/12 h dark cycle, and 40−50 ft candles of light intensity. Food (NIH-07 Open Formula pellets; Zeigler Brothers, Gardners, PA) and water (Columbus Municipal Supply) were provided ad libitum. Animals were identified by tail tattoo after distribution into study groups based on weight and gender using the randomization algorithm in Xybion.

Study Design. Oral administration was chosen because it is the most likely route of human exposure; gavage administration was chosen because of the unpalatability of MEG in the feed at concentrations of 500 ppm or greater, and the volatility of MEG reduces the concentration over time due to evaporation. Doses for this chronic study were selected based on the results from a 14-week subchronic study (Abdo et al., 1999), which used doses ranging from 10 to 1000 mg/kg/day. Doses of 300 mg/kg/day or greater produced possibly life-threatening effects in rodents, whereas doses of 30 mg/kg/day or lower produced no toxicologically significant effects. Groups of 50 animals/sex/species received MEG in 0.5% methylcellulose by gavage at doses of 37, 75, or 150 mg/kg/day, 5 days/week for 105 weeks (rats) or 104 weeks (mice); groups of 60 rats/sex and 50 mice/sex that received 0.5% methylcellulose only served as the vehicle control. A stop-exposure study group of rats (60/sex) received MEG at 300 mg/kg/day in 0.5% methylcellulose by gavage for 53 weeks followed by administration of the vehicle only (0.5% methylcellulose) for the remaining 52 weeks of the study. At 6 and 12 months, 5 rats/sex/time interval, from the control and 300 mg/kg/day dose group, were necropsied. The tissues were examined microscopically. In addition, 10 rat/sex/group and 10 mice/sex/group were bled for MEG plasma determinations and toxicokinetic studies. In addition, an aged animal toxicokinetic study was conducted using 12−15 male and female rats and mice, which were given a single gavage dose of MEG at 150 (rats) or 75 mg/kg (mice) at 18 months of age.

Dose Preparation and Analysis. Prior to study start, dose formulations were shown to be homogeneous and stable for at least 35 days when stored at RT in amber glass bottles with minimal headspace. At monthly intervals, MEG dose formulations were prepared in 0.5% methylcellulose at concentrations calculated from the dose (mg/kg) and dosing volumes (5 mL/kg for rats and 10 mL/kg for mice). Dose formulations were analyzed approximately every 8 weeks, and all formulations as well as samples taken from formulations after dosing animals were within 10% of the target concentrations.

In-Life Data Collection. The animals were observed twice daily (a.m. and p.m.) for mortality and morbidity. Signs of poor health or abnormal behavior were recorded. At monthly intervals, each animal was removed from its cage and examined for unusual or abnormal signs of toxicity. Body weights were recorded at study start, at monthly intervals thereafter, and at study termination. In-life data were collected using NTP's Toxicology Data Management System.

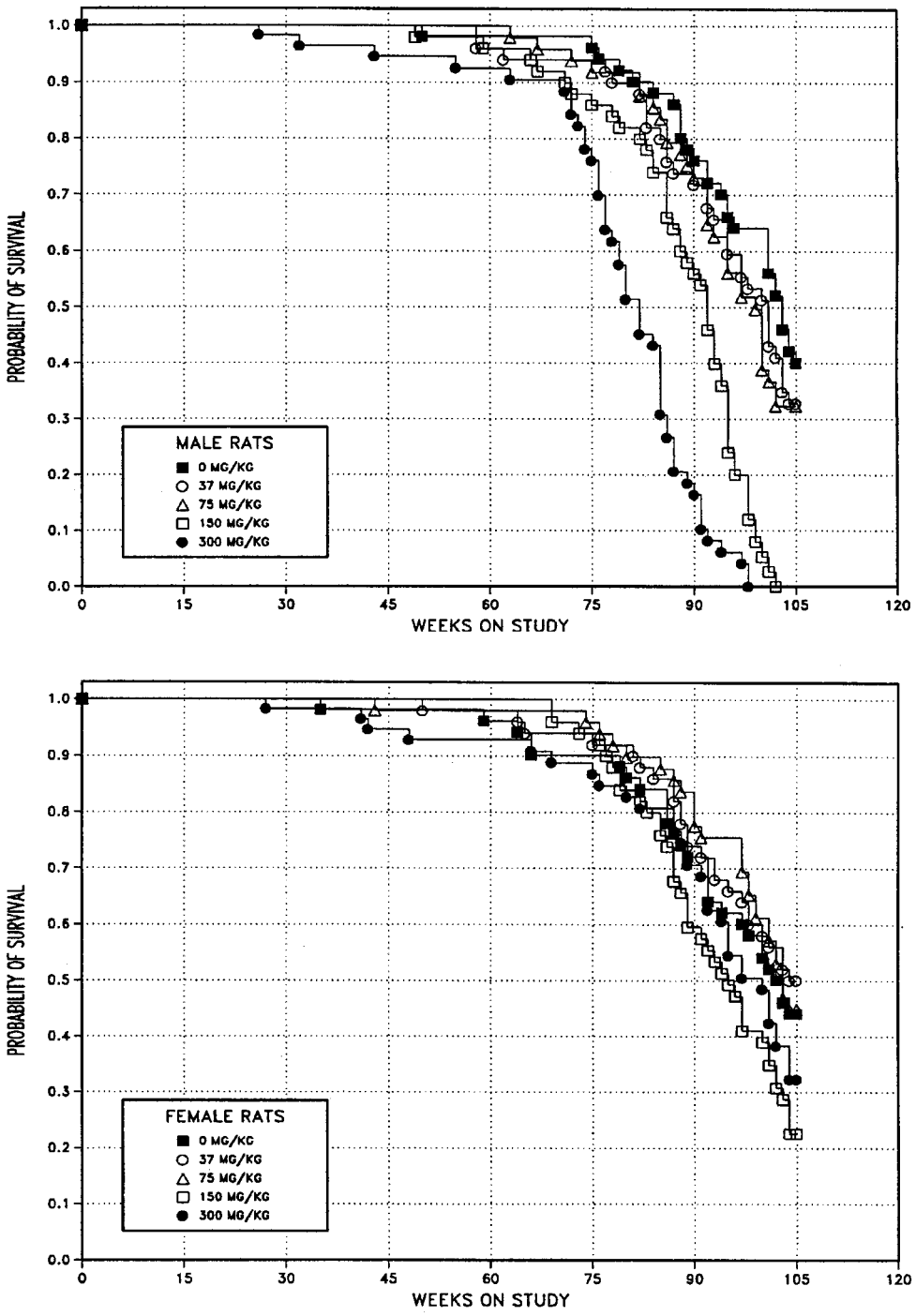

Figure 1. Kaplan−Meier survival curves for male and female F344/N rats administered MEG by gavage for 2 years.

Toxicokinetic Analyses. Blood samples (approximately 1 mL) were collected from the retro-orbital sinus of rats at 6, 12, and 18 month and by cardiac puncture from mice at 12 month. Blood was collected into EDTA-containing tubes (anticoagulant) at 7−10 post-dosing time points from 2 or 3 animals/sex/group/species. Two samples were collected from each rat and one from each mouse. No samples were pooled prior to analysis. Blood was collected at 5 min after dosing (first time point for all collections) to 120, 450, 600, and 780 min after dosing for the 37, 75, 150, and 300 mg/kg/day groups, respectively, at the rat 6- and 12-month interim collections; to 240, 360 and 600 min after dosing for the 37, 75, and 150 mg/kg/day groups, respectively, at the rat 18-month interim collection; and to 75, 150, and 300 min after dosing (male mice) and 60, 150, and 240 min after dosing (female mice) for the 37, 75, and 150 mg/kg/day groups, respectively, at the 12-month collection. Samples were placed in wet ice for less than 60 min before the red cell fraction was separated from the plasma by centrifugation. The plasma was stored at −20 °C until MEG analysis. An HPLC method was used to analyze the samples for MEG (Graves and Runyon, 1995). Analysis of the plasma concentration−time profiles was based on the plasma concentration−time profile and area under the curve (AUC), which was calculated from time 0 to the last time point using the trapezoidal rule.

Pathology. A complete necropsy and microscopic examination were performed on all rats and mice that died early or were killed at scheduled termination, with the exception of those animals used for the toxicokinetic evaluation. Animals were killed by asphyxiation with carbon dioxide. Animals were necropsied in a random order and within 5 min of death. At necropsy, all organs and tissues were examined for grossly visible lesions. All major tissues were fixed and preserved in 10% neutral buffered formalin, trimmed and processed, em-

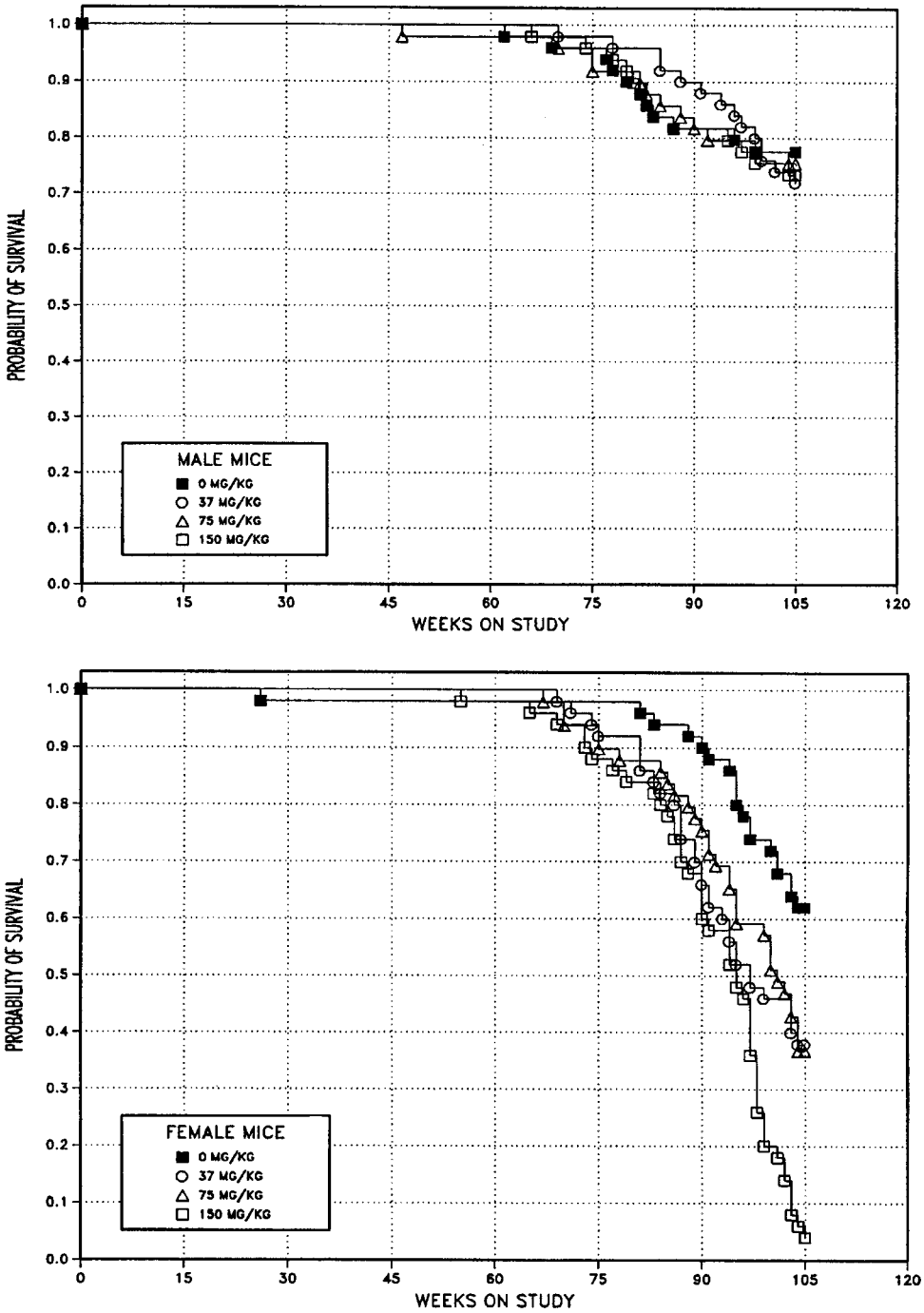

Figure 2. Kaplan−Meier survival curves for male and female B6C3F$_1$ mice administered MEG by gavage for 2 years.

bedded in paraffin, sectioned to a thickness of 5−6 μm, and stained with hematoxylin and eosin for microscopic examination. For all paired organs, samples from each organ were examined. All pathology data were collected and summarized using the TDMS system.

Statistical Analyses. The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. *p* values for survival analyses are two sided. The Poly-*k* test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess the prevalence of neoplasms and nonneoplastic lesions. This test is a survival-adjusted quantal-response procedure that modifies the Cochran−Armitage linear trend test to take survival differences into account. The risk weight value for a given animal was one if the animal had a lesion at that site or if it survived until terminal sacrifice. If the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the *k*th power. Unless otherwise stated, $k = 3$ in the analysis of the site specific lesions (Bailer and Portier, 1988). Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of lesion incidence, and reported *p* values are one sided. For neoplasms and nonneoplastic lesions detected at the interim evaluation, the Fisher exact test (Gart et al., 1979) was used.

RESULTS

Survival. For rats, the percent probability of survival significantly decreased ($p < 0.001$) for the male 150 and

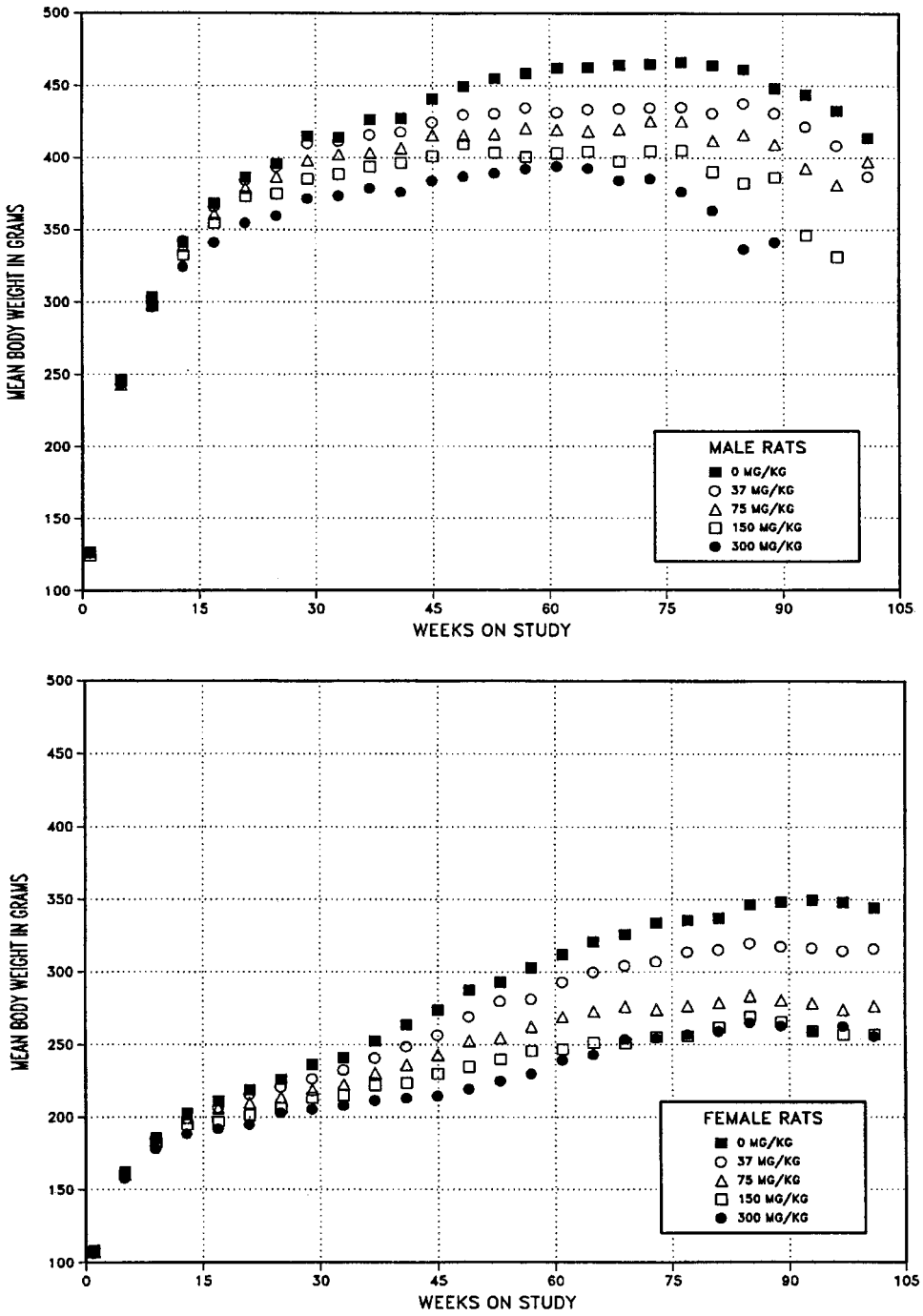

Figure 3. Growth curves for male and female F344/N rats administered MEG by gavage for 2 years.

300 mg/kg/day groups; all male rats in these groups died by study week 97 and 89 (Figure 1). The female rat 150 mg/kg/day group percent probability of survival approached a statistically significant decrease ($p = 0.053$). For mice (Figure 2), while the percent probability of survival to study completion for the male treated groups was similar to control, all female treated groups exhibited a significant decrease ($p \leq 0.013$).

Body Weight Determinations. Terminal group mean body weight values of treated rats and mice decreased in a dose-dependent fashion when compared to their respective control groups (Figures 3 and 4). For rats, group mean body weight values at study end decreased 12, 23, and 26% for the males and 20, 26, and 26% for the female 75, 150, and 300 mg/kg/day groups, respectively. For mice, terminal group mean body weight values decreased 10, 16, and 13% (males) and 39, 44, and 46% (females) relative to control for the 37, 75, and 150 mg/kg/day groups, respectively.

Toxicokinetics. At the rat 6-month interim evaluation period, a proportional increase in AUC was observed when the dose was increased from 37 to 75 mg/kg/day (Table 1). However, increasing the low dose 4 times (150 mg/kg/day) or 8 times (300 mg/kg/day) resulted in a greater than proportional increase in AUC for the male rats, i.e., 8- and 19-fold, respectively, but a slightly less than proportional increase in AUC for the female rats, i.e., 2- and 6-fold, respectively. At 12 months, the greater than proportional increase in AUC from 37 to 150 or 300 mg/kg/day became even more disparate for the males, i.e., 13- and 37-fold, respectively, while the increase for the females was un-

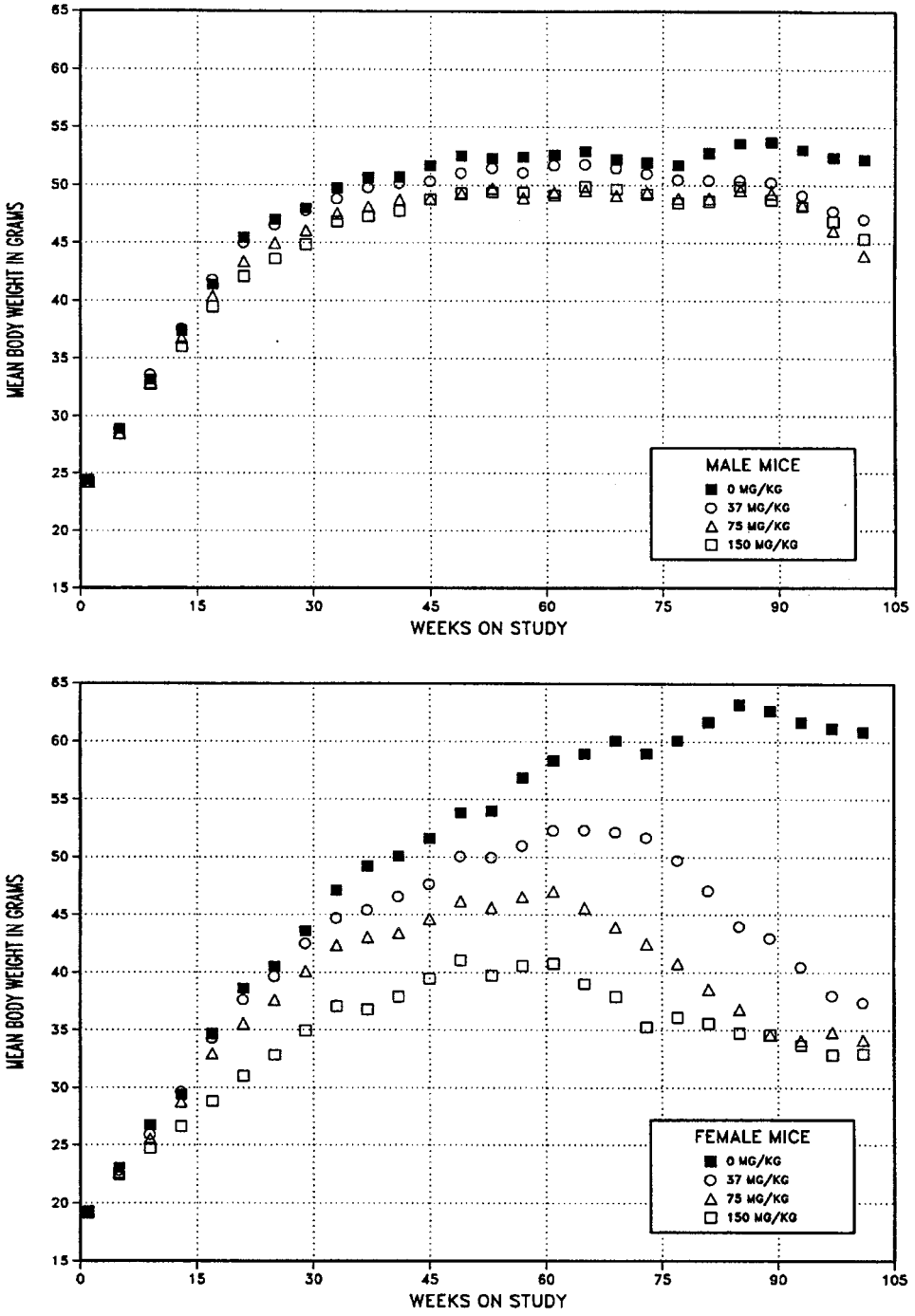

Figure 4. Growth curves for male and female B6C3F1 mice administered MEG by gavage for 2 years.

changed, i.e., 3- and 6-fold, respectively. Likewise, the mouse 12-month AUCs increased more than proportionally (11-fold) from 37 to 150 mg/kg/day for the male mice, whereas a proportional increase in AUCs were measured for the female mice.

For a given dose group, while the AUC values changed somewhat from one interim evaluation period to another, the changes were no more than approximately 2−3-fold. Likewise, a comparison of AUCs between the sexes for a given dose and interim evaluation period, indicated that the AUCs differed by no more than approximately 2−3-fold.

Rat Anatomical Pathology. MEG-related neoplastic and nonneoplastic lesions were found in the liver, glandular stomach and kidney.

Rat 6- and 12-Month Interim Evaluations. The incidences of neoplastic and nonneoplastic liver and glandular stomach lesions are shown in Table 2. At 12 month, there was a significant increase ($p \leq 0.05$) in hepatocellular adenomas (includes multiple) for the male 300 mg/kg/day rats. Although not a statistically significant increase, multiple hepatocellular adenomas (2/5 males), hepatocholangiocarcinoma (1/5 each sex), and glandular stomach neuroendocrine cell hyperplasia (2/5 males, 1/5 females) were also observed after 12 months. The incidence of glandular stomach atrophy was significantly increased ($p \leq 0.01$) for male and female 300 mg/kg/day group rats at both interim evaluations.

Rat 2-Year Evaluation. In general, the incidences and multiplicity of hepatocellular adenoma, carcinoma, and

Table 1. Area under the Curve (AUC) Findings for F344/N Rats at the 6-, 12-, and 18-Month Interim Evaluations and B6C3F$_1$ Mice at the 12-Month Interim Evaluation during the 2-Year Gavage Study of Methyleugenol

species	interim evaluation period	dose group (mg/kg/day)	male AUCs (μg/mL/h)	female AUCs (μg/mL/h)
F344/N rats	6-month	37	0.40	0.55
		75	0.82	1.34
		150	3.11	1.35
		300[b]	7.57	3.24
	12-month	37	0.39	0.71
		75	1.03	1.74
		150	4.96	2.11
		300[b]	14.3	4.26
	18-month	37	0.80	0.77
		75	2.55	0.81
		150	3.92	1.92
		300[b]	*a*	*a*
B6C3F$_1$ mice	12-month	37	15.1	28.3
		75	45.0	56.6
		150	167.9	123.2

[a] No blood samples collected for analysis. [b] Exposure to MEG was stopped after 52 weeks.

adenoma/carcinoma (combined) showed positive trends, and the incidences of multiple hepatocellular adenomas and/or carcinomas were increased in most dose groups of males and females (Table 3). Also found were hepatocholangioma (one 150 mg/kg/day male) and hepatocholangiocarcinomas (one 75 and 150 mg/kg/day male and three 150 mg/kg/day females), although the incidences were not statistically significant. The 300 mg/kg/day male and female groups had a statistically significant increase ($p \leq 0.01$) in the incidence of adenomas, carcinomas, cholangiomas (females only), and cholangiocarcinomas. Numerous types of nonneoplastic lesions were observed in the livers of treated rats, and the incidence and severity of these lesions generally increased with increasing dose. The incidences of eosinophilic foci in all dosed groups of rats and of mixed cell foci in the 37, 75, and 150 mg/kg/day males and in the 75 mg/kg/day females were significantly greater ($p \leq 0.01$) than those in controls. The incidence of basophilic foci showed a negative trend for the male 150 and 300 mg/kg/day groups but were statistically decreased ($p \leq 0.01$) for the female 150 and 300 mg/kg/day groups. The incidences of hepatocyte hypertrophy and oval cell hyperplasia (except for the male 37 mg/kg/day group) were significantly increased. The incidence of cystic degeneration increased significantly for the males at 75 mg/kg/day and higher and the female 300 mg/kg/day group, and the incidence of bile duct hyperplasia was significantly increased for the male 37, 75, and 150 mg/kg/day groups and the female 150 and 300 mg/kg/day groups.

For the glandular stomach, lesions included benign and malignant neuroendocrine tumors, glandular epithelial atrophy, and neuroendocrine cell hyperplasia, which were confined to the fundic region and were more prevalent and severe in females than males (Table 4). The incidences of benign and malignant neuroendocrine tumors were increased in the 150 and 300 mg/kg/day males, and the incidence of malignant neuroendocrine tumors in the 150 mg/kg/day males was significantly increased as compared to the vehicle control group. There was a positive trend in the incidences of benign or malignant neuroendocrine tumors (combined) in females, and the incidences in females administered 75 mg/kg/day or greater were significantly greater than those in the vehicle controls.

In addition, exposure to MEG resulted in significant increases in neoplasms at four other sites (Table 5) including renal tubule adenoma or carcinoma, malignant mesothelioma, mammary gland fibroadenoma, skin fibroma, and skin fibroma or fibrosarcoma (combined).

Mouse Anatomical Pathology. MEG-related increases in the incidences of hepatic neoplasms and nonneoplastic lesions occurred in male and female mice (Table 6). In all dosed groups of mice, the incidences of hepatocellular adenoma and hepatocellular adenoma or carcinoma (combined) were significantly greater than in the vehicle controls. In 37 and 75 mg/kg/day male

Table 2. Incidence (Severity) of Neoplastic and Nonneoplastic Lesion Findings at the 6- and 12-Month Interim Evaluations of F344/N Rats in the 2-Year Gavage Study of Methyleugenol

		6-month		12-month	
organ/finding	sex	vehicle control	300 mg/kg/day[a]	vehicle control	300 mg/kg/day[a]
no. examined	M	5	5	5	5
	F	5	5	5	5
		Liver			
oval cell, hyperplasia	M	0	5** (1.8)[b]	0	5** (1.4)
	F	0	5** (1.0)	0	5** (1.8)
hypertrophy	M	0	5** (3.0)	0	5** (3.0)
	F	0	5** (1.0)	0	5** (3.0)
degeneration, cystic (focal)	M	0	0	0	5** (1.0)
basophilic focus	M	0	3	1	3
	F	0	2	5	3
eosinophilic focus	M	0	3	0	5**
	F	0	0	0	3
mixed cell focus	M	0	5**	0	5**
	F	0	3	0	5**
adenoma (+ multiple)	M	0	0	0	4*
	F	0	0	0	0
		Glandular Stomach			
atrophy	M	0	5**	0	5**
	F	0	5**	0	5**

[a] Exposure to MEG was stopped after 52 weeks. [b] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test. **($p \leq 0.01$).

Table 3. Incidence (Severity) of Neoplastic and Nonneoplastic Lesions from the Livers of F344/N Rats in the 2-Year Gavage Study of Methyleugenol

finding	sex	vehicle control	37 mg/kg/day	75 mg/kg/day	150 mg/kg/day	300 mg/kg/day[a]
no. examined	M	50	50	50	50	50
	F	50	50	49	49	50
bile duct, hyperplasia	M	36 (2.0)[b]	17** (1.2)	16** (1.6)	17** (1.8)	28 (1.7)
	F	11 (1.3)	11 (1.3)	17 (1.3)	22** (1.5)	30** (1.8)
oval cell, hyperplasia	M	14 (2.0)	17 (1.5)	24* (1.5)	34** (1.7)	27** (1.6)
	F	1 (1.0)	15** (1.3)	19** (1.5)	35** (2.2)	34** (2.1)
hypertrophy	M	0	13** (1.7)	25** (2.7)	30** (2.8)	26** (2.5)
	F	1 (2.0)	13** (2.1)	16** (3.1)	26** (3.2)	31** (3.3)
degeneration, cystic (focal)	M	4 (1.3)	2 (1.0)	25** (1.3)	38** (2.2)	41** (2.2)
	F	0	0	1 (1.0)	4 (1.5)	29** (1.9)
basophilic focus	M	23	21	22	7	6
	F	36	36	29	17**	10**
eosinophilic focus	M	11	28**	43**	47**	39**
	F	10	20**	27**	31**	37**
mixed cell focus	M	1	7**	14**	8**	2
	F	6	4	19**	9	7
adenoma, multiple	M	0	5*	14**	24**	24**
	F	0	0	5*	23**	36**
adenoma (+ multiple)	M	5	12*	23**	38**	32**
	F	1	8*	11**	33**	43**
carcinoma, multiple	M	0	0	1	11**	23**
	F	0	0	0	2	9**
carcinoma (+ multiple)	M	2	3	14**	25**	36**
	F	0	0	4	8**	22**
cholangioma	M	0	0	0	1	3
	F	0	0	0	0	8**
cholangioma (+ multiple)	M	0	0	0	1	6**
cholangiocarcinoma (+ multiple)	M	0	0	1	1	7**
	F	0	0	0	3	9**

[a] Exposure to MEG was stopped after 52 weeks. [b] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Poly-3 test. **($p \leq 0.01$).

Table 4. Incidence (Severity) of Neoplastic and Nonneoplastic Lesions in the Glandular Stomachs of F344/N Rats in the 2-Year Gavage Study of Methyleugenol

finding	sex	vehicle control	37 mg/kg/day	75 mg/kg/day	150 mg/kg/day	300 mg/kg/day[a]
no. examined	M	50	50	50	50	50
	F	50	50	50	50	50
atrophy	M	0	14** (1.6)[b]	32** (1.7)	37** (2.2)	29** (2.2)
	F	3 (1.0)	41** (1.6)	45** (2.1)	39** (2.4)	33** (2.2)
neuroendocrine cell, hyperplasia	M	0	0	1 (1.0)	8** (1.5)	8** (1.8)
	F	0	5* (1.0)	11** (2.0)	9** (2.2)	3 (2.0)
benign neuroendocrine tumor	M	0	0	0	3	2
	F	0	1	13**	9**	5*
malignant neuro-endocrine tumor	M	0	0	0	4*	2
	F	0	1	12**	26**	36**

[a] Exposure to MEG was stopped after 52 weeks. [b] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Poly-3 test. **($p \leq 0.01$).

Table 5. Incidence (Severity) of Neoplastic and Nonneoplastic Lesions in F344/N Rats in the 2-Year Gavage Study of Methyleugenol

finding	sex	vehicle control	37 mg/kg/day	75 mg/kg/day	150 mg/kg/day	300 mg/kg/day[a]
no. examined	M	50	50	50	50	50
	F	50	50	50	50	50
nephropathy	M	50 (2.8)	46 (2.4)[b]	48 (2.8)	50 (3.0)	47 (3.3)*
renal tubule adenoma or carcinoma	M	4	2	6	6	8*
renal tubule, hyperplasia focal	M	10 (3.2)	13 (1.8)	20** (2.7)	20** (2.9)	21** (2.7)
malignant mesothelium	M	1	3	5	12**	5
mammary gland fibroadenoma	M	5	5	15**	13**	6
skin fibroma	M	1	9**	8*	5	4
skin fibroma or fibrosarcoma (combined)	M	1	12**	8**	8**	- -

[a] Exposure to MEG was stopped after 52 weeks. [b] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test. **($p \leq 0.01$).

mice and in all dosed groups of females, the incidences of hepatocellular carcinoma were significantly increased. Additionally, the multiple incidences of hepatocellular adenoma in dosed males and females and of hepatocellular carcinoma in dosed females were significantly increased. The incidence of hepatoblastoma in the male 150 mg/kg/day group was slightly greater than control, whereas in females there was a significant, dose-related increase in the incidences of hepatoblastoma.

MEG-related increases in the incidences of neoplasms and nonneoplastic lesions of the glandular stomach occurred in male and female mice (Table 7). Malignant

Table 6. Incidence (Severity) of Neoplastic and Nonneoplastic Lesions of the Liver from B6C3F$_1$ Mice in the 2-Year Gavage Study of Methyleugenol

finding	sex	vehicle control	37 mg/kg/day	75 mg/kg/day	150 mg/kg/day
no. examined	M	49	50	50	50
	F	50	50	49	50
bile duct, hyperplasia	F	1 (2.0)[a]	1 (2.0)	11** (1.5)	9** (1.8)
oval cell, hyperplasia	M	0	8** (1.1)	27** (1.2)	46** (1.5)
	F	0	46** (1.9)	36** (1.9)	38** (1.9)
hypertrophy	M	0	1 (3.0)	7** (2.3)	46** (2.8)
	F	0	10** (2.1)	7** (2.3)	23** (3.0)
hematopoietic cell proliferation	F	4 (1.3)	14** (1.8)	23** (1.7)	24** (1.6)
pigmentation hemosiderin	F	0	11** (1.6)	24** (1.8)	19** (1.6)
inflammation, chronic active	M	19 (1.3)	21 (1.3)	28* (2.3)	28* (1.3)
necrosis	F	5 (2.0)	9 (2.3)	16** (2.3)	17** (2.0)
eosinophilic focus	M	10	20*	25**	19**
adenoma, multiple	M	13	33**	33**	29**
	F	8	39**	38**	32*
adenoma (+multiple)	M	26	43**	38**	39**
	F	20	48**	46**	41**
carcinoma, multiple	M	1	3	3	2
	F	0	24**	29**	38**
carcinoma (+multiple)	M	10	20*	19*	9**
	F	7	37**	47**	47**
hepatoblastoma (+multiple)	M	0	0	1	3
	F	0	6**	11**	15**

[a] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Poly-3 test. **($p \leq 0.01$).

Table 7. Incidence (Severity) of Neoplastic and Nonneoplastic Lesions in the Glandular Stomachs of B6C3F$_1$ Mice in the 2-Year Gavage Study of Methyleugenol

finding	sex	vehicle control	37 mg/kg/day	75 mg/kg/day	150 mg/kg/day
no. examined	M	49	48	49	50
	F	45	49	46	45
atrophy	M	0	3 (1.0)[a]	35** (1.5)	45** (2.6)
	F	0	0	10** (1.6	10** (2.0)
ectasia	M	13 (1.0)	25* (1.0)	40** (1.4)	40** (1.7)
	F	14 (1.0)	33** (1.1)	31** (1.3)	38** (1.4)
hyperplasia	M	0	1 (1.0)	15** (1.7)	20** (1.8)
	F	0	1 (1.0)	5* (1.6)	2 (1.5)
inflammation, chronic active	M	10 (1.0)	11 (1.1)	25** (1.2)	33** (1.3)
	F	17 (1.1)	21 (1.0)	12 (1.0)	14 (1.1)
malignant neuroendocrine tumor	M	0	0	0	2

[a] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. *Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test. **($p \leq 0.01$).

neuroendocrine tumors were observed in two 150 mg/kg/day male mice; one male in this group had a carcinoma.

DISCUSSION

Principal findings attributed to MEG included reduced survival rates, decreased mean body weight gain and marked liver, glandular stomach, and kidney (rat only) lesions in the rats and mice at higher doses. The decreased survival rates of male 150 and 300 mg/kg/day group rats and all dose groups of female mice as well as the dose-related decreases in mean body weight gain of rats and mice were attributed to liver and glandular stomach neoplasms in the rats and liver neoplasms in the mice. Toxic effects to the liver may cause a derangement in protein, carbohydrate, or fat metabolism with subsequent depression in body weight gain. Methyleugenol caused hypoproteinemia and hypoalbuminemia in a 14-week dosed-feed study (Abdo et al., 1999). Decreases in serum proteins can be caused by several factors, including impaired hepatic protein synthesis (Kaneko, 1989; Nguyen, 1989). In addition, MEG induced glandular stomach atrophy, characterized by loss of parietal and chief cells, which likely contributed to the depression in body weight gain. Parietal cells produce gastric acid and chief cells produce proenzymes such as pepsinogen (Guyton and Hall, 1997). The loss of parietal cells leads to hypochlorhydria, increased pH, and decreased activity of acid-activated digestive enzymes such as pepsin, trypsin, and chymotrypsin, which in turn leads to inefficient utilization of food and, consequently, body weight depression. Furthermore, the loss of chief cells reduces pepsinogen production, which also contributes to inefficient food utilization. These toxic effects of MEG in the liver and glandular stomach were irreversible as indicated by persistence of neoplasms and nonneoplastic lesions in the 300 mg/kg/day stop-exposure group rats.

The high lipophilicity and the extremely rapid absorption of MEG may explain the toxicity of the chemical in the liver and glandular stomach of rats and mice. The toxicity of these organs may have been exacerbated by the instant high dose delivery of MEG using gavage administration. In addition, the octanol−water partition coefficient for MEG was estimated at 800, thereby indicating that the chemical is highly lipophilic and able to diffuse through cell membranes relatively quickly. Furthermore, the toxicokinetic data for rats and mice

showed that the time to achieve (T_{max}) maximum concentration in the blood (C_{max}) occurred very early after dosing (5−15 min). Because maximum blood concentrations were achieved so quickly and long before the stomach could have emptied, it is concluded that the chemical was absorbed directly from the stomach and/or forestomach. Absorption was likely hastened by the damaging effects of MEG to the glandular stomach. The rapid absorption of MEG suggests that the chemical was transported in a bolus dose via the portal vein to the liver, thereby enhancing possible damage to the liver. In the liver, MEG is metabolized by the cytochrome P_{450} system (Borchert et al., 1973). Hepatic metabolism involves O-demethylation, side chain hydrolysis, and epoxide diol formation (Solheim and Scheline, 1976). Of the metabolites formed, the two reactive metabolites, 1′-hydroxymethyleugenol and the epoxide diol, were most likely to be responsible for the toxic effects in the liver. Like the hepatocarcinogen safrole, MEG showed DNA-binding activity in in vitro rat liver slices capable of both phase I and phase II activations (unpublished NTP report). The adduct-forming activity of MEG may have been a contributing factor to its hepatocarcinogenic activity.

MEG increased the incidences of liver neoplasms in rats and mice. The exact histogenesis of liver neoplasms remains unresolved. Some recent literature suggests that a putative stem cell is likely to play an important role in the development of some liver neoplasms (Sell and Dunsford, 1989). Oval cells are considered to represent or arise from the stem cells and are thought to have the potential to develop into hepatocytes and/or biliary epithelial cells (Factor et al., 1994). Oval cell proliferation was significantly increased in rats and mice. Some rat liver tumorigens cause an increase in a relatively pure population of hepatocellular neoplasms, while others cause an increase in both hepatocellular and cholangiolar neoplasms (Maronpot et al., 1991). It is possible that the spectrum of neoplasms induced by MEG represents something in between. The proliferative lesions observed in the studies are considered related to MEG. Liver lesions induced by MEG in rats and mice included cytologic alteration, bile duct hyperplasia, necrosis (mice), foci of hepatocellular alteration, hepatocyte hypertrophy, and oval cell hyperplasia. The oval cell hyperplasia and hepatocellular hypertrophy observed in rats at 6 and 12 month persisted until the end of the stop-exposure study, suggesting that these lesions are irreversible. Similarly, for the stop-exposure group, the incidences of liver neoplasms observed after the administration of MEG was discontinued was greater than that observed at 12 month or in the control groups at 2 years. These results suggest that the effect of MEG on the liver was not only irreversible but also that the preneoplastic lesions produced are likely to progress and produce neoplasms in the absence of continued chemical exposure.

Robust liver neoplasm responses to chemical exposure will frequently increase the incidence of hepatoblastomas in mice (Maronpot et al., 1999). While the increases are usually most pronounced in male mice, MEG caused a more pronounced response in the female mice. Hepatoblastomas in the mouse are uncommon neoplasms that occur spontaneously or are chemically induced in the liver of several strains. (Turusov et al., 1973; Nonoyama et al., 1988) including the $B6C3F_1$ mouse used in NTP studies. It is considered a malignant neoplasm, and in NTP studies, its metastatic potential appears similar to that of hepatocellular carcinomas. The cell or origin of the hepatoblastoma has not been clearly defined in rodents or humans, but it may be a very primordial cell (Abenoza et al., 1987; van Eyken et al., 1990; Stocker, 1994). The NTP considers the combinations of hepatocellular carcinoma or hepatoblastoma and of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma to be the most important in analyses evaluating the carcinogenic potential of an agent on the liver.

The finding that MEG included hepatic neoplasms is consistent with findings observed for other chemicals structurally similar to MEG; these chemicals include estragole, safrole, and isosafrole, which also induce neoplasms in rats and mice (IARC, 1976; Miller et al., 1983; CCRIS, 1998). In an earlier study, MEG induced hepatic neoplasms in mice (Miller et al., 1983). Eugenol was not a hepatocarcinogen (Miller et al., 1983), and an NTP study gave equivocal results (NTP, 1983). Miller et al., (1983) showed that the 1′-hydroxy metabolites of these chemicals are more potent hepatocarcinogens than the parent compounds. Studies from the same laboratory demonstrated the importance of sulfation in the hepatotumorigenicity of the 1′-hydroxy metabolite (Boberg et al., 1983). These researchers found that the tumorigenicity of 1′-hydroxysafrole can be inhibited by sulfotransferase inhibitors and can be markedly reduced in brachymorphic mice deficient in xenobiotic sulfation enzymes. Apparently, the sulfur conjugate of the 1′-hydroxy metabolite is the major electrophilic and tumor initiating metabolite.

Stomach lesions were also attributed to MEG exposure. Malignant neuroendocrine tumors were considered related to MEG exposure because they have not been observed previously in NTP gavage studies, occurred with a positive trend in rats and male mice, and were significantly increased in dosed rats. In the forestomach, although the increase in the incidence of squamous cell papilloma or carcinoma (combined) in the 150 mg/kg/day group female rats was not significantly different from that of the vehicle controls, the incidence was outside the historical vehicle control range for corn oil gavage studies. Thus, the increase in the incidence of these neoplasms may have been related to MEG exposure.

The increased incidences of neuroendocrine tumors were also generally associated with a significant dose-related increase in the incidences of glandular stomach atrophy and neuroendocrine cell (enterochromaffin-like cell) hyperplasia. Gastric atrophy was observed in both the 14-week (Abdo et al., 1999) and 2-year studies. Atrophy was characterized by decreased height thinning of the fundic mucosa due to loss of parietal and/or chief cells. As mentioned previously, the loss of parietal cells results in decreased gastric acid secretion. Reduced acid production and increased pH in the stomach are known to be a stimulus for gastrin production. Many reports have shown that long-term exposure to inhibitors of gastric acid secretion results in the induction of enterochromaffin-like tumors (Poynter and Selway, 1991; Johnson et al., 1993; Thake et al., 1995); the magnitude of the proliferative response varies with the compound. For the most part, compounds in these reports are considered nongenotoxic and have a direct inhibitory effect on gastric parietal cells, which results in decreased hydrochloric acid production.

In a study of butachlor (Thake et al., 1995), atrophy of the fundic mucosa with loss of parietal cells occurred in rats along with hypochlorhydria and hypergastrinemia. The neuroendocrine tumors of the glandular stomach induced by this chemical were considered the result of long-term gastrin stimulation of the enterochromaffin-like cells (Thake et al., 1995). In the 14-week studies of MEG (Abdo et al., 1999), there was evidence of inflammation, cellular degeneration, and necrosis in addition to gastric mucosal atrophy, suggesting that parietal cell cytotoxicity may have preceded mucosal atrophy. Because fundic mucosal atrophy was observed in the current studies of MEG, it is probable that hypochlorhydria and hypergastrinemia played a role in the neuroendocrine cell proliferative response. While these factors suggest that the trophic action of gastrin may be the driving force behind the neuroendocrine tumors, MEG has some genotoxic activity that should also be considered when attempting to determine the pathogenesis of the neuroendocrine proliferative response.

In addition to hepatic and gastric neoplasms, MEG caused increases in the incidences of kidney neoplasms (males), malignant mesothelioma, mammary gland fibroadenoma, subcutaneous fibroma (males), and subcutaneous fibroma or fibrosarcoma (combined). Because the incidences of these neoplasms occurred with dose-related trends or were markedly increased in certain dosed groups at rates greater than the historical control rates observed for other routes of exposure, the increases were considered to be related to MEG administration. For the kidney tumors, since the tumors were found in male rats only and male rat kidney tumors are generally associated with $\alpha_{2\mu}$-globulin (Baetcke et al., 1991), a possible mechanism may involve globulin formation as a contributing factor. The possible mechanisms of tumorigenesis of MEG in the other organs are unknown.

The toxicity observed in other organs was considered secondary to MEG exposure. For example, the decreased eosinophilic granularity (cytoplasmic alteration) in the submandibular salivary gland in dosed rats is considered secondary to the effect of MEG on the glandular stomach. Dietary factors such as deprivation of protein are known to result in the loss of zymogen granules (McBride et al., 1987). The administration of MEG to rats may have resulted due to loss of parietal and chief cells of the glandular stomach. Secretion of gastric acid and its production of protein-digesting enzymes are the function of parietal and chief cells, respectively. Therefore, loss of these cells could have inhibited proper protein utilization and, hence, lead to protein deficiency. Likewise, there were increased incidences of bone marrow hyperplasia in dosed rats and mice, hematopoietic proliferation in the liver of mice, and hyperplasia of the adrenal glands of rats, which were considered to be secondary effects of the increased incidences of necrosis and inflammation associated with the large and multiple liver neoplasms in dosed animals.

In conclusion, the findings of the present study indicate that the no-observable-effect level for MEG is below 37 mg/kg/day. Under the conditions of these 2-year gavage studies, there was clear evidence of carcinogenic activity of MEG in male and female F344/N rats based on the increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach in male and female rats and the increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma and subcutaneous fibroma and fibroma or fibrosarcoma (combined) in male rats. A marginal increase in squamous cell neoplasms of the forestomach may have been related to MEG administration in female rats. There was clear evidence of carcinogenic activity of MEG in male and female B6C3F$_1$ mice based on the increased incidences of liver neoplasms in males and females. Neuroendocrine tumors of the glandular stomach in male mice were also considered related to exposure to MEG. Finally, in addition to the neoplastic lesions, MEG administration caused significant increases in nonneoplastic lesions of the liver and glandular stomach.

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