NTP
National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6C3F1/N Mice

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to NTP. The studies described in the NTP Technical Report series are designed and conducted to characterize and evaluate the toxicological potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in NTP Technical Reports are derived solely from the results of these NTP studies, and extrapolation of the results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's carcinogenic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and the Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP Technical Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Explanation of Levels of Evidence of Carcinogenic Activity

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, in as much as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major flaws **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised on March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear** evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- Some evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- Equivocal evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- No evidence of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- Inadequate study of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been

adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

Peer Review

The members of the Peer Review Panel who evaluated the draft *NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6C3F1/N Mice* on December 12, 2019, are listed below. Panel members served as independent scientists, not as representatives of any institution, company, or governmental agency. A summary of the Peer Review Panel's comments is provided in Appendix G.

In this capacity, panel members had five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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Abstract

2-Hydroxy-4-methoxybenzophenone (2H4MBP) is approved by the U.S. Food and Drug Administration for use in sunscreens and other personal products in concentrations of up to 6% either alone or in combination formulations and as an indirect food additive in acrylic and modified acrylic plastics that come into contact with food. 2H4MBP was nominated to the National Toxicology Program by the National Cancer Institute due to widespread exposure via sunscreen use and lack of carcinogenicity data. 2H4MBP was also nominated by a private individual to ascertain genotoxic potential. Male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats (after weaning) and B6C3F1/N mice were exposed to 2H4MBP (greater than 99% pure) in feed for 2 years. Perinatal studies and 14-week interim evaluations were also conducted in rats. Genetic toxicology studies were conducted in *Salmonella typhimurium* and *Escherichia coli*.

Two-year Study in Rats

Beginning on gestation day (GD) 6, groups of 42, 35, 35, and 43 F_0 time-mated female rats were fed diets containing 0, 1,000, 3,000, and 10,000 ppm 2H4MBP, respectively, for 39 days. Groups of 50 (1,000 and 3,000 ppm) or 60 (0 and 10,000 ppm) F_1 rats per sex continued on study after weaning and were fed diets containing the same exposure concentrations for 105 weeks; 10 F_1 rats per sex from the 0 and 10,000 ppm groups were evaluated at 14 weeks. Dietary concentrations of 1,000, 3,000, and 10,000 ppm resulted in average daily doses of approximately 58, 168, and 585 mg 2H4MBP/kg body weight for males and 60, 180, and 632 mg/kg for females.

Survival of all exposed groups of F_1 male and female rats was not significantly different from that of the control groups. Over the course of the study, mean body weights of F_1 males and females in the 10,000 ppm exposure groups were 10–25% lower than those of the control groups. After week 77, F_1 female mean body weights in the 3,000 ppm exposure group were 10% lower than those of the control group. Feed consumption by exposed groups of F_1 males and females was generally similar to that by the control group throughout the study.

In the brain, the occurrence of malignant meningiomas in males at the end of the 2-year study was 0/50, 1/50, 3/50, and 0/50. One male in the 3,000 ppm group had a malignant meningioma in the spinal cord.

In the thyroid gland, the incidence of C-cell adenoma in 3,000 ppm females was significantly greater than that in the control group at the end of the 2-year study.

In the uterus, the incidence of stromal polyp in 3,000 ppm females was significantly increased. A significantly increased incidence of atypical endometrium hyperplasia of the uterus also occurred at 3,000 ppm; however, the incidence of adenocarcinoma was significantly decreased in this group.

In the adrenal cortex, the incidences of focal hypertrophy were significantly increased in 1,000 and 3,000 ppm females at the end of the 2-year study.

In the testes, the incidence of interstitial cell hyperplasia occurred with a positive trend at the end of the 2-year study.

Two-year Study in Mice

Groups of 50 male and 50 female mice were fed diets containing 0, 1,000, 3,000, or 10,000 ppm 2H4MBP (equivalent to average daily doses of approximately 113, 339, and 1,207 mg 2H4MBP/kg body weight for males and 109, 320, and 1,278 mg/kg for females) for 104 (females) or 105 (males) weeks. Survival of all exposed groups of male and female mice was not significantly different from that of the control groups. Mean body weights of 1,000 and 3,000 ppm males and females were within 10% of those of the control groups throughout the study. Mean body weights of 10,000 ppm males and females were at least 10% lower than those of the control groups generally after weeks 17 and 12, respectively. Feed consumption by exposed groups of males and females was not significantly different from that by the control groups.

The incidences of pigment in the bone marrow were significantly increased in 10,000 ppm males and females. The incidences of pigment in the spleen were significantly increased in 10,000 ppm males and 3,000 and 10,000 ppm females.

In the liver, the incidence of hepatocyte syncytial alteration was significantly increased in all exposed groups of males.

In the kidney, the incidence of renal tubule cytoplasmic alteration was significantly increased in 10,000 ppm males. The incidence of osseous metaplasia was significantly increased in 10,000 ppm females compared to the control group.

Genetic Toxicology

Results of bacterial mutagenicity tests conducted using standard testing approaches with the same lot of 2H4MBP tested in the 2-year studies were negative in TA98 and TA100, as well as in *Escherichia coli* strain WP2 *uvr*A pKM101, with and without rat liver S9.

Conclusions

Under the conditions of these 2-year studies, there was *equivocal evidence of carcinogenic activity* (see Explanation of Levels of Evidence of Carcinogenic Activity) of 2H4MBP exposure in male Hsd:Sprague Dawley® SD® rats based on the occurrence of malignant meningiomas in the brain. There was *equivocal evidence of carcinogenic activity* in female Hsd:Sprague Dawley® SD® rats based on the increased incidence of thyroid C-cell adenomas and the increased incidence of uterine stromal polyps. There was *no evidence of carcinogenic activity* in male or female B6C3F1/N mice at exposure concentrations of 1,000, 3,000, and 10,000 ppm.

Increases in the incidences of nonneoplastic lesions of the testis in male rats and of the uterus and adrenal cortex in female rats occurred with exposure to 2H4MBP. Increases in the incidences of nonneoplastic lesions of the bone marrow (males and females), spleen (males and females), kidney (males and females), and liver (males) in mice occurred with exposure to 2H4MBP.

Synonyms: Benzophenone-3; (2-hydroxy-4-methoxyphenyl)-phenylmethanoneoxybenzone; oxybenzone

Summary of the Perinatal and Two-year Carcinogenesis and Genetic Toxicology Studies of 2-Hydroxy-4-methoxybenzophenone

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
Concentrations in Feed	0, 1,000, 3,000, or 10,000 ppm	0, 1,000, 3,000, or 10,000 ppm	0, 1,000, 3,000, or 10,000 ppm	0, 1,000, 3,000, or 10,000 ppm
Survival Rates	30/50, 29/50, 24/50, 33/50	30/50, 33/50, 34/50, 26/50	34/49, 40/50, 43/50, 42/50	42/50, 39/50, 44/50, 46/50
Body Weights	10,000 ppm group: ~10% less than the control group after week 69; 20% lower than the control group at study end	10,000 ppm group: ~10% less than the control group after week 17; ~16% lower than the control group after week 45; 24% lower at study end 3,000 ppm group: ~10% less than the control group after week 77	10,000 ppm group: 10% less than the control group after week 17	10,000 ppm group: 10% less than the control group after week 12
Nonneoplastic Effects	Testis: interstitial cell, hyperplasia (1/50, 0/50, 0/50, 5/50)	Uterus: endometrium, atypical hyperplasia (9/50, 14/50, 19/50, 14/50) Adrenal cortex: hypertrophy, focal (24/50, 42/50, 39/50, 27/50)	Bone marrow: pigment (3/47, 2/48, 9/48, 50/50) Spleen: pigment (4/48, 5/50, 10/49, 17/50) Liver: hepatocyte, syncytial alteration (2/49, 39/50, 45/50, 48/50) Kidney: renal tubule, cytoplasmic alteration (0/48, 0/50, 0/50, 46/50)	Bone marrow: pigment (6/49, 0/50, 0/50, 50/50) Spleen: pigment (12/49, 10/50, 36/49, 38/50) Kidney: metaplasia, osseous (0/49, 1/50, 3/50, 5/50)
Neoplastic Effects	None	None	None	None
Equivocal Findings	Brain: malignant meningioma (0/50, 1/50, 3/50, 0/50)	Thyroid gland: C-cell adenoma (5/50, 11/50, 17/50, 10/50) Uterus: stromal polyp (8/50, 15/50, 18/50, 10/50)	None	None
Level of Evidence of Carcinogenic Activity	Equivocal evidence	Equivocal evidence	No evidence	No evidence

Genetic Toxicology

Bacterial Gene Mutations: Negative in *Salmonella typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2 *uvr*A pKM101, with and without S9

Overview

The National Toxicology Program (NTP) has assessed the potential adverse effects of sunscreens in human-relevant model systems; the data presented herein are part of that larger effort. The scope of 2-hydroxy-4-methoxybenzophenone (2H4MBP) studies includes the assessment of potential endocrine activity in the U.S. Environmental Protection Agency Endocrine Disruptor Screening Program Phase 1 studies (summary results of these data are provided in Appendix F), and characterization of the potential effects of continuous 2H4MBP exposure over multiple generations using the NTP Modified One-Generation study design. In this study design, exposure to 2H4MBP in the diet began on gestation day (GD) 6. At weaning, one and two pups per sex per litter were allocated to prenatal and reproductive performance cohorts, respectively. In addition to an assessment of reproductive performance, F_2 fetal outcomes (GD 21 fetal examinations) were assessed in one cohort and the potential effects on parturition and early growth of the F_2 generation were assessed in the other cohort. Internal dose metrics were also assessed. Apical indicators sensitive to endocrine modulation were measured (e.g., anogenital distance, thoracic nipple retention, pubertal indices, reproductive tissue histology). The U.S. Food and Drug Administration's National Center for Toxicological Research (NCTR), in partnership under an Interagency Agreement, has also examined the effects of maternal and lactational exposure to 2H4MBP on development and reproductive organs in male and female rat offspring, and on transcriptional changes in the testes and prostates of young rats. NCTR is also conducting fertility, embryo-fetal, and pre- and postnatal rat studies to characterize the potential effects of 2H4MBP exposure. NTP previously conducted 2- and 13-week toxicity studies by dermal and oral routes and assessed the genotoxic potential of 2H4MBP. Potential effects of 2H4MBP exposure on mouse reproduction were assessed using the Reproductive Assessment by Continuous Breeding protocol.

Introduction

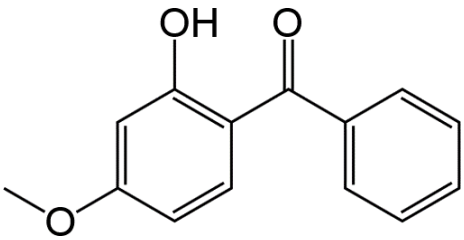


Figure 1. 2-Hydroxy-4-methoxybenzophenone (CASRN 131-57-7; Chemical Formula: $C_{14}H_{12}O_3$; Molecular Weight: 228.25)

Synonyms: Benzophenone-3; (2-hydroxy-4-methoxyphenyl)-phenylmethanoneoxybenzone; oxybenzone.

Chemical and Physical Properties

2-Hydroxy-4-methoxybenzophenone (2H4MBP) is an off-white to light-yellow powder with a melting point of 62°C to 65°C. 2H4MBP is relatively insoluble in water (69 mg/kg at 25°C) and is readily soluble in most organic solvents. 2H4MBP absorbs ultraviolet (UV) A (320 to 400 nm) and UVB (290 to 320 nm) light and is photostable.[1]

Production, Use, and Human Exposure

2H4MBP is synthesized by condensation of benzoic acid with resorcinol monomethyl ether in the presence of heat, zinc chloride, and polyphosphoric acid or by the Friedel-Crafts reaction of benzoyl chloride with 3-hydroxyanisole.[2]

2H4MBP is commonly used in sunscreens and other personal care products at concentrations of up to 6% to protect the wearer from solar erythema. Per the Environmental Working Group's Guide to Sunscreens database,[3] 2H4MBP is found in more than 1,000 products, including beach, sport, and baby sunscreens (619), moisturizers with SPF (150), and lip balms (109), often in combination with other sunscreens. 2H4MBP has also been combined with the insecticide repellent N,N-diethyl-meta-toluamide (DEET).[4] 2H4MBP is also used as a photostablizer for synthetic resins and polymers, including plastics (0.05–0.5%) to prevent UV degradation.[5-7] Exposure can occur when present in acrylic and modified acrylic plastics that come in contact with food.[8]

2H4MBP and its metabolites are typically excreted in urine. A study using National Health and Nutrition Examination Survey (NHANES) cycle data from 2004 to 2012 demonstrated more than 96% of the 10,232 samples (representing all populations) contained measurable urinary concentrations of 2H4MBP. Creatinine-adjusted urinary least square geometric mean concentrations ranged from 9 to 17 ng/mL in males, and 18 to 45 ng/mL in females. Children and adolescent concentrations ranged from 17 to 27 and 13 to 24 ng/mL, respectively.[9; 10] Higher urinary concentrations of 2H4MBP were observed in non-Hispanic whites and have been attributed to increased sunscreen use (28 versus 13 ng/L).[11] Higher concentrations in females have been ascribed to the use of personal care products (e.g., lip balms, cosmetics) that often contain 2H4MBP.[11]

Regulatory Status

2H4MBP is approved by the U.S. Food and Drug Administration (FDA) for use as a sunscreen when present up to 6% either alone or in combination formulations and as an indirect food additive in acrylic and modified acrylic plastics that come into contact with food.[12; 8] 2H4MBP is also approved for use as a nonfood inert pesticide additive.[13] Section 8(a) of the Toxic Substances Control Act requires manufacturers of 2H4MBP to report preliminary assessment information concerned with production, exposure, and use to the U.S. Environmental Protection Agency.

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

2H4MBP was well absorbed (≥63.9%) following a single gavage administration of [^{14}C]2H4MBP (3.01 to 2,570 mg/kg) in male F344/N rats, with the administered dose excreted primarily via urine (63.9% to 72.9%) and feces (19.3% to 41.7%) by 72 hours postadministration. The radioactivity remaining in tissues 72 hours after administration was low (~0.1%) in all dose groups.[14] Following dermal application of 51.6, 204, and 800 μg [^{14}C]2H4MBP (in ethanol) in male rats, the dose was excreted mainly via urine (32.4%, 39.2%, and 13.2%) and feces (16.9%, 22.2%, and 9.15%) by 72 hours postapplication. The dose excreted in urine and feces indicated that the applied dose absorbed was 49.3%, 61.4%, and 22.4%, respectively, for 51.6, 204, and 800 μg [^{14}C]2H4MBP. When the dose (50 μg) was applied in a lotion vehicle, the dose absorbed (51.8%) was similar to that in ethanol with 33.9% and 17.9% of the dose recovered in urine and feces, respectively.[14]

Absorption, distribution, metabolism, and excretion (ADME) were also investigated in male and female Sprague Dawley rats and B6C3F1/N mice following gavage administration or dermal application of [^{14}C]2H4MBP.[15] Following a single gavage administration (10, 100, or 500 mg/kg [^{14}C]2H4MBP) in rats, most of the administered dose was excreted in urine (53% to 58%) and feces (38% to 42%) by 72 hours postadministration with no observable sex difference in excretion. The radioactivity in urine indicated that ≥53% of the administered dose was absorbed. Following a single 100 mg/kg gavage dose in male mice, urinary (≥34%) and fecal (≥24%) excretion was similar to that of rats. However, mice (5% to 15%) excreted a higher percentage of administered dose as exhaled CO_2 compared to rats (~1%). The retention of dose in tissues was low at 72 hours (<1%) in all gavage groups.

ADME of 2H4MBP was investigated in rats and mice at 72 hours following dermal application of 0.1 or 10 mg/kg [^{14}C]2H4MBP formulated in several vehicles. In male rats, the highest absorption was observed following application in light paraffin oil (80%). Absorption following application in ethanol, ethanol:coconut oil (1:1), or coconut oil was comparable to paraffin oil (64% to 73%). In contrast, the absorption of 2H4MBP from the lotion vehicle (olive oil:emulsifying wax:water [15:15:70 v:v:v]) in male (10 mg/kg, 46%) and female (15 mg/kg, 29%) rats was lower relative to other vehicles. Both male and female mice absorbed approximately 60–69% of 10 mg/kg dose in ethanol or acetone and 37–46% of 10 mg/kg dose when formulated in the lotion vehicle. There was no dose-related effect in absorption (0.1 versus 10 mg/kg) in either male rats or mice.[15]

Kinetics of disposition of 2H4MBP has been investigated in rats in limited studies. Following a single gavage dose of 100 mg/kg 2H4MBP in male Sprague Dawley rats, the time (T_{max}) to reach the maximum concentration, C_{max} (21.21 μg/mL), was 3 hours; the elimination of 2H4MBP in plasma was biphasic with alpha and beta half-lives of 0.88 and 15.9 hours, respectively. Of the tissues examined, the liver had the highest concentration of 2H4MBP and conjugated 2H4MBP at 6 hours.[16] In another study, following a 100 mg/kg gavage dose in male Sprague Dawley rats, similar T_{max} (2.72 hours) and C_{max} (21.21 μg/mL) were observed, with a plasma elimination half-life of 4.58 hours.[17] Following a single gavage dose of 10 mg/kg in male and female Harlan Sprague Dawley rats, T_{max} and C_{max} were 6.0 hours and 8.5 ng/mL, respectively, for males and 2.3 hours and 2.9 ng/mL for females. The plasma elimination half-life for males was 6.4 hours and for females was 18.5 hours. The bioavailability of 2H4MBP in male and female rats was <1%, demonstrating extensive first-pass metabolism of 2H4MBP following gavage administration.[15]

2H4MBP was metabolized via numerous pathways in rodents, including demethylation, oxidation, glucuronidation, and sulfation. Products identified in bile and/or urine of rodents following administration of 2H4MBP were 2H4MBP, 2,4-dihydroxybenzophenone (DHB), 2,3,4-trihydroxybenzophenone (THB), 2,5-dihydroxy-4-methoxybenzophenone (D2H4MBP), and their corresponding glucuronide and sulfate conjugates (Figure 2).[14; 16; 15; 18] Similar metabolites were also observed in vitro following incubation of 2H4MBP with microsomes.[19; 20] 2H4MBP and DHB have been quantified in serum from pregnant rats.[21] In a recent National Toxicology Program (NTP) study, rats were exposed in utero and postnatally to 0, 3,000, 10,000, and 30,000 parts per million (ppm) 2H4MBP in the diet, and plasma concentrations of free (unconjugated analytes) and/or total (free and all conjugated forms) 2H4MBP, DHB, THB, and D2H4MBP were quantified (Figure 2).[22] Free D2H4MBP and THB were not detected in plasma. Mean plasma concentrations of total 2H4MBP and DHB were higher (~100- to 300-fold) than the free 2H4MBP and DHB concentrations demonstrating extensive conjugation of 2H4MBP and its metabolites. The rank order of the total concentrations were 2H4MBP ≈ DHB > D2H4MBP >> THB. Free and total analyte plasma concentrations were not sex-dependent in either PND 28 or PND 56 pup plasma.

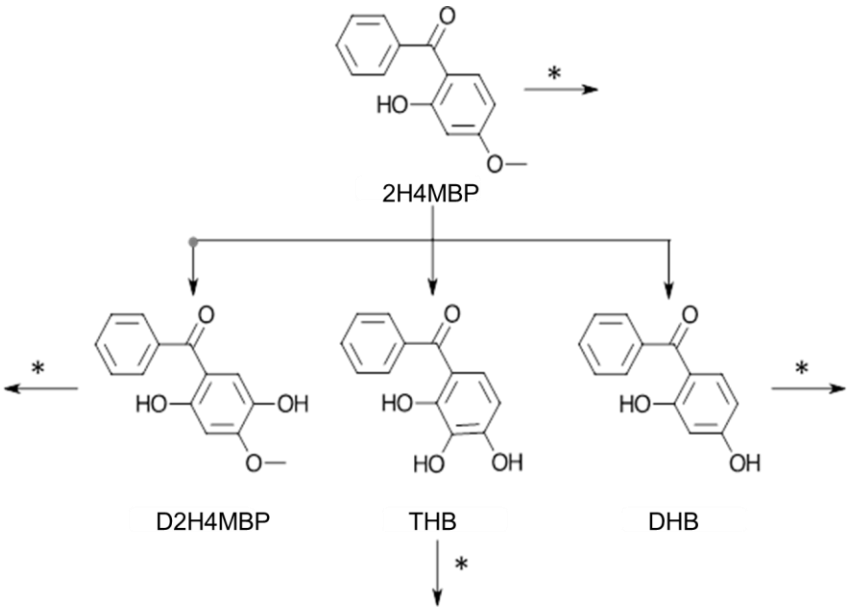


Figure 2. Metabolism of 2-Hydroxy-4-methoxybenzophenone in Rodents

2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; D2H4MBP = 2,5-dihydroxy-4-methoxybenzophenone; THB = trihydroxybenzophenone; DHB = dihydroxybenzophenone.
*Indicates glucuronide and sulfate conjugates.

Humans

ADME data on 2H4MBP in humans are limited. Human studies with sunscreens have demonstrated that 2H4MBP is readily absorbed from the skin.[23] A study that used excised human epidermis in Franz diffusion cells showed that approximately 10% of the dermally applied dose of 2H4MBP is absorbed.[24] When applied dermally, 2H4MBP and the metabolites DHB and 2,2'-dihydroxy-4-methoxybenzophenone can be detected in serum and are excreted in urine.[25; 26] A study examining the absorption of 2H4MBP, and subsequent irradiation with UV A and B rays, demonstrated that participants excreted 1.2–8.7% (mean 3.7%) of the total applied dose in the urine. 2H4MBP could be detected in the urine 3–5 days after application. UV irradiation did not affect the amount of 2H4MBP excreted.[27] Frequency of sunscreen use is also related to urinary 2H4MBP concentrations with frequent users having much higher urinary concentrations.[28] 2H4MBP has been detected in maternal urine and breast milk.[29; 30]

Toxicity

Experimental Animals

The acute rat dermal LD_{50} has been reported to be greater than 16 g/kg body weight. Concomitant local skin reactions consisting of mild to moderate erythema were observed in the absence of significant pathologic findings.[6] The rat oral LD_{50} for 2H4MBP has been reported to be greater than 12.8 g/kg.[31] These authors also reported that administration of 0.5% or 1% 2H4MBP in rat diet for 12 weeks was associated with growth depression. Upon examination at week 6, female rats exposed to 0.5% or 1% displayed a leukocytosis with an increase in the lymphocyte count and a decrease in the neutrophil count, as well as a decrease in hemoglobin concentration. At week 12, rats exposed to either concentration displayed anemia and

lymphocytosis with a reduction in granulocytes. The weights relative to brain of the pituitary gland, thymus, heart, adrenal gland, lung, and spleen were also reduced in both sexes. The 0.5% females showed an increase in the relative weight to brain of the thyroid gland and first stages of kidney degeneration. Degenerative nephrosis was diagnosed both macro- and microscopically in the kidneys of both sexes at 1%.

NTP has reported the findings of studies conducted in F344 rats and B6C3F1 mice exposed to: (1) 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm 2H4MBP in feed for 2 or 13 weeks; (2) 0, 1.25, 2.5, 5, 10, or 20 mg/kg body weight for 2 weeks dermally in acetone or lotion; and (3) 12.5, 25, 50, 100, or 200 mg/kg body weight in acetone or lotion for 13 weeks duration.[32] In rats and mice, dietary administration of 6,250 ppm and higher concentrations of 2H4MBP for 2 weeks were associated with increases in liver weights and with marked hepatocyte cytoplasmic vacuolization.

In the 13-week rat dietary study, 50,000 ppm of 2H4MBP was associated with lower male and female body weight gains. Liver weights were increased at 3,125 ppm and higher, whereas kidney weights were increased at the 6,250 ppm and higher dietary exposure concentrations. Males and females in the 50,000 ppm exposure groups also displayed lower weights of the heart, lungs, and thymus, and males in the 50,000 ppm exposure group displayed lower weights of the brain and testis.[33] Histopathologic kidney findings primarily occurred in high-exposure rats, and included dilated tubules, tubular epithelial cell regeneration, papillary degeneration or necrosis, and inflammation. Although cytoplasmic vacuolization was not observed in the liver, liver enzymes remained elevated at 13 weeks.

In the 13-week mouse dietary study, there was an exposure-related decrease in body weight gains, mild increases in liver weights in both sexes, and variable increases in kidney weight in females. Histopathological findings were limited to kidneys of mice receiving 25,000 and 50,000 ppm and included eosinophilic protein casts in dilated renal tubules, and mild inflammation. In the 2-week rat and mouse dermal studies, small and variable increases in liver and kidney weights were observed primarily in the higher dose groups. In the 13-week dermal studies, kidney weights were increased in dosed groups of female rats and variably increased in dosed male mice. These weight changes were not associated with any histopathological changes.[33] A 4-week dermal study in rats using 100 mg/kg of 2H4MBP in petroleum jelly twice a day did not affect body weight; liver, kidney, or testes weight; or histopathology.[34] 2H4MBP exposure lowered rat blood glutathione-*S*-transferase levels.

Humans

Other than the human findings summarized in the Immunotoxicity section of this report, no other significant toxicity studies in humans were identified in the public domain.

Reproductive and Developmental Toxicity

Models of Endocrine Activity

2H4MBP has been reported to bind to and activate estrogen receptor alpha (ERα) with an IC_{50} ranging from 3 to 20 × 10^{-6} M.[35-38] 2H4MBP can also activate estrogen receptor beta (ERβ),[39; 37] and reports indicate that 2H4MBP can act as ERα, ERβ, and progesterone receptor antagonists.[35; 39; 37] In NTP-sponsored ER binding and activation studies conducted under OPPTS 890.1250 and

OPPTS[a] 890.1300, maximal mean specific binding was >75% (more than 75% of the radiolabeled estradiol remained bound), which categorizes 2H4MBP as "non-interacting"; however, 2H4MBP was able to induce a luciferase response, albeit weak (>10%, log EC_{50}s −3.2 and −4.0 M) (Appendix F).[40; 41] 2H4MBP acts as an estrogen in stimulating MCF7 cell proliferation (IC_{50} 3.4×10^{-6}). 2H4MBP has been shown to induce a uterotrophic response (ED_{50}: 1,000 to 1,500 mg/kg per day) in immature rats.[42] However, 2H4MBP did not cause a uterotrophic response in ovariectomized rats when tested up to 1 g/kg in an NTP study (Appendix F). 2H4MBP was evaluated in quantitative (dose-response) high throughput screening assays by NTP in the Toxicology in the 21st Century (Tox21) program, and significant activity was observed in assays measuring stimulation of ER, progesterone receptor, constitutive androstane receptor, pregnane X receptor, retinoic acid receptor, and estrogen-related receptor signaling pathways. In addition, 2H4MBP was shown to inhibit androgen receptor signaling (https://pubchem.ncbi.nlm.nih.gov/compound/4632#section=BioAssay-Results&fullscreen=true).

2H4MBP exposure in male rainbow trout and Japanese medaka has been shown to induce vitellogenin production, an estrogenic response, and change the number of eggs produced and egg viability/hatching.[43] 2H4MBP has also been shown to increase plasma concentrations of testosterone in male adult Japanese medaka and to decrease the estradiol to testosterone ratio in both male and female fish with concomitant downregulation of gonadal steroidogenic genes (*star*, *Cyp11a*, *Cyp17*, *Hsd3b*, *Hsd17b3*, and *Cyp19a*).[44]

Experimental Animals

The potential for 2H4MBP exposure to affect sperm density and vaginal cytology has been reported.[32] Rats and mice received 0, 3,125, 12,500, or 50,000 ppm in the diet for 90 days. Male rats exposed to 50,000 ppm weighed 30% less than control animals and displayed lower epididymis (17%) and caudal epididymis (22%) weights and sperm density (27%). Female rats displayed an increase in estrous cycle length in the 12,500 and 50,000 ppm groups (>1 day).

High-exposure male mice displayed a 27% decrease in sperm density and weighed 16% less than control mice. High-exposure female mice displayed a slight increase in estrous cycle length relative to control mice (>0.5 day). NTP conducted a Reproductive Assessment by Continuous Breeding (RACB) study in mice at exposure concentrations of 12,500, 25,000, and 50,000 ppm in the diet.[45] 2H4MBP had no effect on F_0 fertility, but the number of live pups per litter was significantly reduced in the 25,000 and 50,000 ppm groups, which was associated with lower parental body weights. There were no changes in sperm density or estrous cyclicity; however, the cumulative days to litter were increased in the 50,000 ppm group. 2H4MBP had minimal effects on fertility in the F_1 generation, but pup weights were significantly reduced. Collectively, it was concluded that 2H4MBP caused systemic toxicity but had minimal effects on fertility and reproduction at the exposure concentrations studied. Another study examined the effects of 0, 10, 20, 100, or 400 mg/kg body weight of 2H4MBP dermally applied to mice for 13 weeks. No

[a]Guidelines issued before April 22, 2010, refer to "OPPTS" because the office name changed from "Office of Prevention, Pesticides and Toxic Substances" to "Office of Chemical Safety and Pollution Prevention," or "OCSPP."

effects on body weight, organ weights, sperm density, or testicular histopathology were attributed to 2H4MBP exposure.[46]

The potential effects of maternal and lactational exposure to 2H4MBP on F_1 development and reproductive organs have been assessed.[21] Sprague Dawley rats received 0, 1,000, 3,000, 10,000, 25,000, or 50,000 ppm 2H4MBP in the diet from gestation day (GD) 6 until weaning on postnatal day (PND) 23. Exposure to 2H4MBP was associated with increased liver and kidney weights in dams. Clinical pathology findings in the dams during GDs 10, 15, and 20 were elevation of glucose, alanine aminotransferase, alkaline phosphatase, cholesterol, total bile acids, and depression of aspartate aminotransferase, blood urea nitrogen, and creatinine. These findings occurred primarily in the higher dose groups and often at all time points. Alanine aminotransferase and cholesterol were elevated in the male and female offspring at the higher two exposure concentrations. No significant differences were observed in littering parameters. Male and female pups in the two highest dose groups displayed lower body weights. Male anogenital distance adjusted for body weight at PND 23 was decreased in the highest dose group. At necropsy on PND 23, female liver weights relative to body weight were higher at exposure concentrations ≥10,000 ppm. In the highest dose group, spermatocyte development was impaired and ovarian follicular development was delayed.

Humans

Maternal 2H4MBP exposure, determined primarily via third trimester urinary concentrations, was associated with lower birth weight of girls and the opposite in boys.[47] In another study, maternal gestational urinary 2H4MBP concentrations were positively associated with body weight and head circumference at birth.[48] Maternal exposure to 2H4MBP has been postulated to be involved in the development of Hirschsprung's disease (incidence of 1 in 5,000 newborns).[49] One hypothesis is that this complex congenital disease is caused by gene–environment interactions that can lead to intestinal obstruction and chronic constipation in the offspring. Pregnant women that have higher 2H4MBP concentrations in urine exhibit a higher odds ratio (2.4 to 2.6:1) of having a child with Hirschsprung's disease.[50]

In the 293T and SH-SY5Y cell migration model of Hirschsprung's disease, 2H4MBP suppressed migration and altered the levels of key migratory proteins at both the ribonucleic acid (RNA) and transcribed protein levels in the absence of cytotoxicity.[51; 50] A study looking at the potential effect of 2H4MBP dermal application and serum hormone changes in young men and postmenopausal women concluded that the amount of 2H4MBP absorbed did not alter the endogenous reproductive hormone homeostasis.[23]

Immunotoxicity

Experimental Animals

A study conducted per procedures outlined by the Federal Hazardous Substances Labeling Act (FSLA) for acute skin irritation and scored for irritation per the Draize method concluded that an occlusive patch containing 0.5 mL or 0.5 mg at 2H4MBP concentrations from 4% to 100% was nonirritating to intact and abraded albino rabbit skin.[6] 2H4MBP at 100% up to 100 mg was found not to be irritating to the rabbit eye using the modified FSLA or Draize methods. A sunscreen containing 6% 2H4MBP was found not to be photosensitizing in albino rabbits and

was negative for sensitization potential in the Klingman Maximization Procedure[6] and local lymph node assay.[52]

Humans

Some reports have indicated that 2H4MBP might induce allergenic and sensitization responses.[6] In a sunscreen sensitization study, researchers detected allergy and/or photoallergy to 2H4MBP in 3.7% of the human subjects, which was attributed to application of moisturizing creams that contained 2H4MBP.[53] A subsequent study sponsored by Schering-Plough HealthCare Products reported the results of the meta-analysis of 64 unpublished studies conducted at 10 independent clinical laboratories representing the results of 19,570 individuals subjected to human repeat insult patch tests and photoallergy studies between 1992 and 2006.[54] These studies were aggregated and analyzed to evaluate the irritancy and sensitization potential of sunscreen products containing 2H4MBP concentrations between 1% and 6%. Forty-eight dermal responses were considered suggestive of sensitization or irritation with a mean rate of response of 0.26%. The authors concluded that sunscreen products formulated with 1% to 6% oxybenzone do not possess a significant sensitization or irritation potential for the general public. 2H4MBP was also negative in an in vitro phototoxicity assay using SkinEthic™, a human epidermis model.[55]

Carcinogenicity

No reports of studies that characterize the potential for 2H4MBP to induce neoplasms were found in the literature.

Genetic Toxicity

2H4MBP was negative in tests screening for mutagenic agents in dental materials[56] and in sunscreens.[57; 58] However, as reported in NTP Toxicity Report 21,[33] 2H4MBP showed weak mutagenic activity in *Salmonella typimurium* strains TA100 and TA97 when tested in the presence of 30% hamster liver S9 mix; results from a second bacterial mutation test that tested the compound with 10% hamster liver S9 mix were negative. 2H4MBP was also positive for induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells when testing occurred in the presence of rat liver S9 mix.[32] The parent structure, benzophenone, also was negative in an NTP bacterial mutagenicity assay in several strains of *Salmonella typhimurium*, with and without exogenous metabolic activation,[59] but both 2,2′- dihydroxy-4-methoxybenzophenone and 4,4′-bis(dimethylamino)benzophenone (Michler's ketone) were mutagenic in bacterial assays conducted by NTP. Michler's ketone also was positive in the mouse lymphoma L5178Y cell mutation assay, and in tests for induction of chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells.[60-64] In vivo assessments of genotoxic potential showed no activity in the *Drosophila* somatic mutation and recombination test following exposure of larva to feed containing 3,500 ppm 2H4MBP, and no induction of chromosomal aberrations in male Sprague Dawley rats treated with up to 5 g/kg 2H4MBP either as a single gavage treatment or after five once-daily gavage treatments.[65] 2H4MBP was also negative for induction of micronucleated erythrocytes in male and female mice treated via dosed feed for 90 days.[32] No activity in any of the Tox21 deoxyribonucleic acid (DNA) damage assays was observed, consistent with the results in standard in vitro and in vivo assays for genotoxicity.

Study Rationale

2H4MBP was nominated to NTP by the National Cancer Institute because of high exposure via use of 2H4MBP-containing sunscreen products and lack of carcinogenicity data. 2H4MBP was also nominated by a private individual to ascertain genotoxic potential. NTP designed 2-year studies in rats and mice to evaluate the potential carcinogenic activity of 2H4MBP. The initiation of F_1 generation exposure on GD 6 (after expected implantation) in the 2-year rat study was selected to reflect potential human exposure to 2H4MBP, often present in sunscreens and cosmetics; exposure may occur at any human life stage, including in utero and early life. As the pattern of disposition is similar following oral and dermal exposure, 2H4MBP exposure via the diet was selected, rather than topical application, to sustain internal exposure. It was also recognized that if applied topically, internal dose would be influenced by intra- and inter-animal grooming behavior.

Materials and Methods

Procurement and Characterization of 2-Hydroxy-4-methoxybenzophenone

2-Hydroxy-4-methoxybenzophenone (2H4MBP) was obtained from Ivy Fine Chemicals Corporation (Cherry Hill, NJ) in one lot (20080801) that was used in the perinatal and 2-year studies. Identity and purity analyses were conducted under the analytical chemistry laboratory and study laboratory at Battelle (Columbus, OH) (Appendix B). Reports on analyses performed in support of the 2H4MBP studies are on file at the National Institute of Environmental Health Sciences.

Lot 20080801 of the chemical, a light-yellow powder, was identified as 2H4MBP by infrared (IR) and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) with mass spectrometry (MS) detection. The IR spectrum was in good agreement with a reference spectrum[66] and the structure of 2H4MBP. Proton and carbon-13 NMR spectra were consistent with computer-predicted spectra and the structure of the test article. The mass spectrum of the major peak from the GC/MS analysis matched a reference spectrum[67] for 2H4MBP.

The purity of lot 20080801 was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and using GC with flame ionization detection (FID). Lot 20080801 was screened for common residual volatile solvents using GC with electron capture detection (ECD) and FID. Differential scanning calorimetry (DSC) was used to determine the purity of the test article. In addition, Karl Fisher titration of lot 20080801 was performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Purity assessment by HPLC/UV found no reportable impurities in lot 20080801. GC/FID analysis yielded a purity of 99.8% and found one impurity with an area of 0.17% of the total peak area. No significant halogenated or nonhalogenated volatile impurities were found in the bulk chemical. Purity by DSC was 99.2%. Karl Fischer analysis indicated that no quantifiable water was present in the test article. The overall purity of lot 20080801 was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature in sealed amber glass containers. Periodic reanalyses of the bulk chemical were performed during the perinatal and 2-year studies by the study laboratory using HPLC/UV and no degradation of the bulk chemical was detected.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared approximately monthly by mixing 2H4MBP with feed (Table B-1). Formulations were stored in sealed amber plastic bags at room temperature for up to 42 to 43 days.

Homogeneity studies of the 1,000 and 10,000 ppm dose formulations in NIH-07 and NTP-2000 feed were performed before the animal studies by the analytical chemistry and study laboratories. Additional homogeneity studies of the 1,000 and 10,000 ppm dose formulations in NTP-2000

feed were performed during the chronic studies by the study laboratory. Stability studies of the 1,000 ppm dose formulation in NIH-07 and NTP-2000 feed were performed by the analytical chemistry laboratory using the same analytical method. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed amber plastic bags at room temperature.

Periodic analyses of the dose formulations of 2H4MBP were conducted by the study laboratory using HPLC/UV (Table B-2; Table B-3). Of the dose formulations analyzed, all were within 10% of the target concentrations; all animal room samples for rats and mice were within 10% of the target concentrations.

Animal Source

Time-mated (F_0) female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Harlan Laboratories (now Envigo, Indianapolis, IN). Male and female B6C3F1/N mice were obtained from Taconic (Taconic Biosciences, Germantown, NY).

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by AAALAC International. Studies were approved by the Battelle Columbus Operations (Columbus, OH) Animal Care and Use Committee and conducted in accordance with all relevant National Institutes of Health (NIH) and National Toxicology Program (NTP) animal care and use policies and applicable federal, state, and local regulations and guidelines.

Two-year Studies

Study Design for Rats

Exposure concentration selection was based in part on Fischer 344/N rat studies reported in Toxicity Report 21.[32] Rats were exposed to 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm 2H4MBP in the diet for up to 13 weeks. After 13 weeks of 2H4MBP exposure, males and females in the 25,000 ppm groups weighed approximately 10% less than rats in the control groups, whereas males and females exposed to 50,000 ppm weighed 32% and 14% less, respectively, than rats in the control groups. Both males and females in the 50,000 ppm groups had increased absolute and relative kidney weights and exhibited renal papillary necrosis and tubule dilatation. Renal tubule dilation was observed in 100% of the 25,000 ppm male rats and in 30% of the males in the 12,500 ppm group. Therefore, the 10,000 ppm exposure concentration was selected for both male and female rats, anticipating that the system would be appropriately challenged and recognizing strain differences might exist.

Beginning on GD 6, groups of 42, 35, 35, or 43 F_0 time-mated female rats were fed diets containing 0, 1,000, 3,000, or 10,000 ppm 2H4MBP, respectively, throughout gestation and lactation. Groups of 50 (1,000 and 3,000 ppm) or 60 (0 and 10,000 ppm) F_1 rats per sex continued on in the study after weaning and were fed diets containing the same respective 2H4MBP concentration for 105 weeks. An interim evaluation, using 10 F_1 rats per sex from the 0 and 10,000 ppm groups, was conducted at 14 weeks. To evaluate transcriptional changes in the liver, liver tissue from five control and five 10,000 ppm F_1 male rats from the 14-week interim

evaluation was collected and processed for microarray analyses at the Battelle Biomedical Research Center (Columbus, OH) (Appendix E).

F_0 female rats were 11 to 14 weeks old upon receipt. Evidence of mating is defined as GD 1; F_0 females were received on GD 2 and held for 4 days. F_0 females were randomly assigned to exposure groups on GD 5. Randomization was performed by a body-weight-partitioning algorithm to produce similar group mean weights (PATH/TOX SYSTEM software, Xybion Medical Systems Co., Cedar Knolls, NJ).

F_0 females were quarantined for 11 days after receipt. Ten nonmated females received with the time-mated females were designated for disease monitoring 11 days after arrival; samples were collected for serological analyses, and the rats were terminated, necropsied, and examined for the presence of disease or parasites. The health of the F_1 rats was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix D). Pinworms (*Syphacia spp.*) were diagnosed in sentinel animals during routine health monitoring evaluations. Infected animals did not display clinical signs and no pathological lesions were noted in relation to the presence of the pinworms. Following this finding, NTP, in coordination with the testing laboratory, developed and implemented a successful plan of pinworm containment and eradication. NTP requires the testing laboratories to actively monitor animals to ensure the continued exclusion of pinworms from all studies going forward. All other test results were negative.

F_0 female rats were housed individually during gestation and with their respective litters during lactation. F_0 females were weighed on GDs 5, 6, 9, 12, 15, 18, and 21 and on PNDs 1, 4, 7, 14, and 21. The day of parturition was considered to be PND 0. Total litter weight was collected on PND 1. Individual F_1 pups were weighed on PNDs 4, 7, 14, and 21. On apparent GD 27, all time-mated female rats that did not deliver were euthanized and the uteruses were examined and stained for evidence of implantation. On PND 1, the number of live and dead F_1 pups, sex ratio, whole litter weights, and litter weights/sex were recorded.

F_1 litters were standardized on PND 4 to eight pups/litter, with at least two pups of each sex and a preference for four males and four females each. Litters that did not meet the minimum of eight pups (or if they had fewer than two pups of either sex) were removed from the study. For continuation of exposure after weaning, two males and two females per litter from 30 litters in the 0 and 10,000 ppm groups and from 25 litters in the 1,000 and 3,000 ppm groups were randomly selected. During gestation and lactation, feed consumption was measured continuously by cage. On the day the last litter reached PND 18, litters were randomly selected and F_1 pups from these litters were randomly selected for the 2-year study. On the day the last litter reached PND 21, the pups were weaned and the dams necropsied, and gross lesions collected. Weaning marked the beginning of the 2-year chronic phase of the study.

F_1 pups were housed two (males) or four (females) per cage. Feed and water were available ad libitum. In the 2-year chronic phase of the rat study, feed consumption in F_1 rats was measured initially, weekly for 13 weeks, then for one 7-day period every 4 weeks, and at the end of the study. Cages were changed weekly through PND 4, then changed every 2 weeks. Racks were changed and rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix C.

Study Design for Mice

Dose selection was based primarily on the 13-week B6C3F1/N mouse data presented in Toxicity Report 21.[32] Mice were exposed to 0, 3,125, 6,250, 12,500, 25,000, or 50,000 ppm 2H4MBP in the diet. After 13 weeks of exposure, male and female mice in the 25,000 ppm groups displayed 14% and 8% lower body weights, respectively, relative to animals in the control groups. Absolute liver weights were higher in both the males and females exposed to ≥6,250 ppm, which was associated with cytoplasmic vacuolization of the hepatocytes. Therefore, 10,000 ppm was selected as the high-exposure concentration for male and female mice.

Groups of 50 mice per sex were fed diets containing 0, 1,000, 3,000, or 10,000 ppm 2H4MBP for 104 (females) or 105 (males) weeks. Mice were quarantined for 14 days prior to study start. Mice were approximately 4 to 5 weeks old upon receipt and were randomly assigned to exposure groups using the same body-weight partitioning algorithm as was used for F_0 female rats to produce similar group mean weights (PATH/TOX SYSTEM software).

Five male and five female mice were randomly selected for parasite evaluation and gross observation of disease. Mice were 6 to 7 weeks old on the first day of the study. The health of the mice was monitored during the study according to the protocol of the NTP Sentinel Animal Program (Appendix D). All test results were negative.

Mice were housed individually (males) or five (females) per cage. Feed and water were available ad libitum. Feed consumption was measured weekly for 13 weeks, then for one 7-day period every 4 weeks, and at the end of the study. Cages were changed once weekly (males) or twice weekly (females) and rotated every 2 weeks. Racks were changed and rotated every 2 weeks.

Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is given in Appendix C.

Clinical Examinations and Pathology

For the 2-year studies in rats and mice, animals were observed twice daily for morbidity and moribundity and were weighed initially, weekly for the first 13 weeks, then at 4-week intervals, and at study termination. Clinical findings were recorded every 4 weeks and at the end of the studies.

Complete necropsies and microscopic examinations were performed on F_1 rats and mice. At the 14-week F_1 rat interim evaluation (control and 10,000 ppm, n = 10/sex/group), the weights of the heart, right kidney, liver, lung, right testis, and thymus were collected. At necropsy, all organs and tissues were examined for grossly visible lesions and all major tissues were fixed and preserved in 10% neutral buffered formalin except for eyes, testes, vaginal tunics, and epididymides, which were first fixed in Davidson's solution or modified Davidson's solution. Spinal cord was only collected from animals that had clinical neurological signs. Tissues were processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin (H&E) for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. In the original evaluation of the uterus, a transverse section through each uterine horn, approximately 0.5 cm cranial to cervix, was collected for histopathology evaluation. During the pathology review process, a residual tissue evaluation of the uterus of rats was performed in which all remaining

uterine tissue, including the cervix, and vaginal tissue was sectioned longitudinally, processed, and examined histologically. Results from the residual uterine evaluation were combined with those from the original, transverse sections of uterus. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment (QA) laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a QA pathologist evaluated slides from all neoplasms and all potential target organs, which included the kidney of rats and mice; the liver of male rats and male and female mice; the adrenal cortex and thyroid gland of rats; the epididymis, heart, pancreas, and testis of male rats; the uterus of female rats (including all the sections made from residual tissue); and the ovary of female mice.

The QA report and the reviewed slides were submitted to the NTP pathologist, who reviewed and addressed any inconsistencies in the diagnoses made by the laboratory and QA pathologist. The QA pathologist, who served as the coordinator of the Pathology Working Group (PWG), presented representative histopathology slides containing examples of lesions related to test agent administration, examples of disagreements in diagnoses between the laboratory and QA pathologist, or lesions of general interest to the PWG for review. The PWG consisted of the NTP pathologist and other pathologists experienced in rodent toxicologic pathology. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, QA pathologist, and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman[68] and Boorman et al.[69] For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of Brix et al.[70]

An additional examination to confirm lesions recorded during the QA process in the longitudinal sections of uterus, cervix, and vagina was undertaken by a pathologist that had not previously examined the tissues.

A special pathology peer review panel was convened to examine all the diagnoses of malignant meningiomas, both in the brain and spinal cord. The consensus on the tumors (both brain and spinal cord) was that they were all malignant meningiomas, confirming the diagnoses made by the study pathologist and confirmed by the QA pathologist. In addition, digital scans of the spinal cord neoplasm were also sent, without knowledge of the chemical or dose group, to eight pathologists, including two neuropathologists. The general consensus on the spinal cord lesion, which included detailed input from the neuropathologists, was that it was a malignant meningioma.

Table 1. Experimental Design and Materials and Methods in the Perinatal and Two-year Feed Studies of 2-Hydroxy-4-methoxybenzophenone

Rats	Mice
Study Laboratory	
Battelle (Columbus, OH)	Same as in rats
Strain and Species	
Sprague Dawley (Hsd:Sprague Dawley® SD®)	B6C3F1/N
Animal Source	
Harlan Laboratories (Indianapolis, IN), now Envigo	Taconic Farms (Germantown, NY)
Time Held before Studies	
F_0 females: 4 days	Males: 16 days
	Females: 15 days
Average Age When Studies Began	
F_0 females: 11 to 14 weeks	6 to 7 weeks
Date of First Exposure	
F_0 females: October 1, 2010	Males: July 16, 2010
F_1: November 8 (males) or 9 (females), 2010	Females: July 15, 2010
Duration of Exposure	
F_0 females: GD 6 to PND 21 F_1 (interim evaluation): 14 weeks	Males: 105 weeks
F_1 (2-year study): 105 weeks	Females: 104 weeks
Date of Last Exposure	
F_0 females: November 8, 2010	Males: July 13, 2012
F_1 (14-week interim evaluation): February 8 (males) or 9 (females), 2011	Females: July 11, 2012
F_1 (2-year study): November 5 to 7 (males) or 7 to 9 (females), 2012	
Necropsy Dates	
F_1 (2-year study): November 5 to 7 (males) or 7 to 9 (females), 2012	Males: July 11–13, 2012
	Females: July 9–11, 2012
Size of Study Groups	
F_0 females: 42 (0 ppm), 35 (1,000 and 3,000 ppm), or 43 (10,000 ppm)	50/sex
F_1: 60/sex (0 and 10,000 ppm) or 50/sex (1,000 and 3,000 ppm)	
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights	Same as in rats

Rats	Mice
Animals per Cage	
F_0 females: 1 (with litter)	Males: 1
F_1: 2 (males) or 4 (females)	Females: 5
Method of Animal Identification	
F_0 females: Cage card and tail marking with permanent pen	Tail tattoo
F_1: Cage card and tail tattoo	
Diet	
Irradiated NIH-07 meal feed (perinatal phase) or irradiated NTP-2000 meal feed (2-year study) (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed twice weekly	Irradiated NTP-2000 meal feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed once (males) or twice (females) weekly
Water	
Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as in rats
Cages	
Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly through PND 4, then twice weekly, rotated every 2 weeks	Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (males) or twice weekly (females), rotated every 2 weeks
Bedding	
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ) changed with cage changes	Same as in rats
Rack Filters	
Spun-bonded polyester (Snow Filtration Co., Cincinnati, OH; National Filter Media, Olive Branch, MS), changed every 2 weeks	Spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks
Racks	
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks	Same as in rats
Animal Room Environment	
Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Same as in rats
Exposure Concentrations	
0, 1,000, 3,000, or 10,000 ppm in feed	Same as in rats

Rats	Mice
Type and Frequency of Observation	
F_0 females: Observed twice daily. Weighed on GDs 5, 6, 9, 12, 15, 18, and 21 and on PNDs 1, 4, 7, 14, and 21. Feed consumption was measured continuously from GD 6 to PND 21. F_1 rats: Observed twice daily. Litter data (litter count by sex, litter weights by sex, and litter observations) were recorded on PND 1. Pups per litter were recorded on PNDs 2 and 3. Pups were weighed on PNDs 4, 7, 14, and 21, weekly for 13 weeks, then every 4 weeks, and at the end of the study. Clinical findings were recorded every 4 weeks beginning at week 6 and at the end of the study. Feed consumption was recorded initially, continuously for 13 weeks, then for one 7-day period every 4 weeks.	Observed twice daily. Weighed initially, weekly for 13 weeks, then every 4 weeks, and at the end of the studies. Clinical findings were recorded at week 5 then every 4 weeks. Feed consumption was measured initially, continuously for 13 weeks, then every 4 weeks for a 7-day period.
Method of Euthanasia	
Euthanasia solution and decapitation (≤PND 10 pups and fetuses); exsanguination (interim rats); carbon dioxide (≥PND 10 pups, F_0 females, and adult F_1 rats)	Carbon dioxide
Necropsy	
Necropsies were performed on all animals. Organs weighed at the 14-week interim evaluation were heart, right kidney, liver, lung, right testis, and thymus.	Necropsies were performed on all animals.
Microarray Analyses	
Liver tissue of five control and five 10,000 ppm F_1 male rats was collected and processed to evaluate transcriptional changes in the liver at 14 weeks (Appendix E).	
Histopathology	
Complete histopathology was performed on all F_1 rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, trachea, urinary bladder, and uterus. An additional extended evaluation of the uterus was performed that included all remaining cervical, vaginal, and uterine tissue remnants.	Complete histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eyes, Harderian gland, gallbladder, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, trachea, urinary bladder, and uterus.

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier[71] and is presented graphically. Animals surviving to the end of the observation period are treated

as censored observations, as are animals dying from unnatural causes within the observation period. Animals dying from natural causes are included in analyses and are treated as uncensored observations. For the two-year mouse study, dose-related trends are identified with Tarone's life-table test,[72] and pairwise dose-related effects are assessed using Cox's[73] method. For the rat perinatal study, dose-related trends and pairwise dose-related effects on survival are assessed using a Cox proportional hazards model[73] with a random litter effect. All reported p values for the survival analyses are two-sided.

Calculation of Incidence

The incidence of neoplasms or nonneoplastic lesions (Appendix H) is presented as the numbers of animals bearing such lesions at a specific anatomic site. For calculation of incidence rates (Appendix H), the denominator for most neoplasms and all nonneoplastic lesions is the number of animals where the site was examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, Zymbal's gland, and spinal cord) before microscopic evaluation, the denominator consists of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominator consists of the number of animals on which a necropsy was performed. Additional study data (Appendix H) also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal euthanasia.

Analysis of Neoplasm and Nonneoplastic Lesion Incidence

Statistical analyses of neoplasm and nonneoplastic lesion incidence considered two features of the data. Some animals did not survive the entire 2 years of the study, so survival differences between groups had to be considered. Also, up to two rats per sex were randomly selected from each litter to participate in the study. The statistical analysis of lesion incidence used the Poly-3 test to account for survival differences, with a Rao-Scott adjustment for litter effects, as described below.

The Poly-k test[74-76] was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to account for survival differences. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is 1 if the animal had a lesion at that site or if it survived until terminal euthanasia; if the animal died prior to terminal euthanasia 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 kth power.

This method yields a lesion prevalence rate that depends only on the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time.[74] Unless otherwise specified, a value of $k = 3$ was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier[74] following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and B6C3F1 mice.[77]

Bailer and Portier[74] showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams.[78] Poly-3 tests used the continuity correction described by Nam.[79]

Littermates tend to be more like each other than like fetuses/pups in other litters. Failure to account for correlation within litters leads to underestimates of variance in statistical tests, resulting in higher probabilities of Type I errors ("false positives"). Because up to two pups per sex per litter were present in the core rat study, the Poly-3 test was modified to accommodate litter effects using the Rao-Scott approach.[80] The Rao-Scott approach accounts for litter effects by estimating the ratio of the variance in the presence of litter effects to the variance in the absence of litter effects. This ratio is then used to adjust the sample size downward to yield the estimated variance in the presence of litter effects. The Rao-Scott approach was implemented in the Poly-3 test as recommended by Fung et al.,[81] formula $\bar{\tau}_{RS2}$.

Tests of significance included pairwise comparisons of each dosed group with control groups and a test for an overall dose-related trend. Continuity-corrected Rao-Scott-adjusted Poly-3 tests were used in the analysis of lesion incidence and reported p values are one-sided. The significance of a lower incidence or decreasing trend in lesions is represented as 1−p with the letter N added (e.g., p = 0.99 is presented as p = 0.01N). For neoplasms and nonneoplastic lesions observed without litter structure (e.g., at the interim evaluation), Poly-3 tests that included the continuity correction, but without adjustment for potential litter effects, were used for trend and pairwise comparisons to the control group.

To evaluate incidence rates by litter, the proportions of litters affected by each lesion type were tested among groups. Cochran-Armitage trend tests and Fisher exact tests[82] were used to test for trends and pairwise differences from the control group, respectively.

Analysis of Continuous Variables

Before statistical analysis, extreme values identified by the outlier test of Dixon and Massey,[83] for small samples (n < 20), and Tukey's outer fences method,[84] for large samples (n ≥ 20), were examined by NTP personnel, and implausible values were eliminated from the analysis. Organ and body weight measurements, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[85] and Williams.[86; 87] Dam gestational and lactational feed consumption, litter sizes, pup survival, implantations, number of resorptions, and proportions of male pups per litter for all rat endpoints were analyzed using the nonparametric multiple comparison methods of Shirley[88] [as modified by Williams[89]] and Dunn[90] given that these endpoints typically have skewed distributions. For all quantitative endpoints unaffected by litter structure, Jonckheere's test[91] was used to assess the significance of the dose-related trends and to determine at the 0.01 level of significance, whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test).

Postweaning body weights were measured on two pups per sex per litter in the 2-year study; more than two pups per sex per litter were possible in preweaning body weight measurements.

The analyses of pup body weights and body weights adjusted for litter size (described below) of these animals took litter effects into account using a mixed model, where litters were the random effect. To adjust for multiple comparisons in these models, a Dunnett-Hsu adjustment was used.[92] Dam body weights during gestation and lactation were analyzed with the parametric multiple comparison procedures of Dunnett[85] and Williams,[86; 87] depending on whether Jonckheere's test indicated the use of a trend-sensitive test. P values for these analyses are two-sided.

Analysis of Gestational and Fertility Indices

Cochran-Armitage trend tests were used to test the significance of trends in gestational and fertility indices across dose groups. The Fisher exact test was used to conduct pairwise comparisons of each dosed group with the control group. P values for these analyses are two-sided.

Body Weight Adjustments

Preweaning pup body weights were adjusted for live litter size as follows: A linear model was fit to body weights as a function of dose and litter size. The estimated coefficient of litter size was then used to adjust each pup body weight on the basis of the difference between its litter size and the mean litter size. Prestandardization PND 4 body weights were adjusted for PND 1 litter size, and body weights measured between PND 4 poststandardization and PND 21 were adjusted for PND 4 poststandardization litter size. Following adjustment, body weights were analyzed with a linear mixed model with a random litter effect.

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical control data must be generally similar. Significant factors affecting the background incidence of neoplasms at a variety of sites are diet, sex, strain/stock, vehicle and route of exposure. The NTP historical control database contains all 2-year studies for each species, sex, and strain/stock with histopathology findings in control animals completed within the most recent 5-year period,[93-95] including the concurrent control groups for comparison across multiple technical reports. Due to the sectioning of residual tissues for the rat uteri, the historical control for this organ consists of three studies at this time: Indole-3-carbinol, perfluorooctanoic acid, and this study. In general, the historical control data for a given study includes studies using the same route of administration, and the overall incidence of neoplasms in control groups for all routes of administration are included for comparison, including the current study.

Quality Assurance Methods

The 2-year studies were conducted in compliance with FDA Good Laboratory Practice Regulations.[96] In addition, the 2-year study reports were audited retrospectively by an independent QA contractor against study records submitted to the NTP Archives. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in

the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

Genetic Toxicology

The genetic toxicity of 2H4MBP was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium* and *Escherichia coli*. The protocol for these studies and the results are given in Appendix A.

The genetic toxicity studies have evolved from an earlier effort by NTP to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term in vitro and in vivo genetic toxicity tests (structure-activity relationships). The short-term tests were originally developed to clarify proposed mechanisms of chemical-induced DNA damage on the basis of the relationship between electrophilicity and mutagenicity[97] and the somatic mutation theory of cancer.[98; 99] However, it should be noted that not all cancers arise through genotoxic mechanisms.

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites.[100] A positive response in the *Salmonella* test was shown to be the most predictive in vitro indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens).[101; 102] Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

Results

Data Availability

The National Toxicology Program (NTP) evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-TR-597.[b]

Rats

Two-year Study

Perinatal Exposure

Gestation body weights of dams receiving 10,000 ppm 2H4MBP in the diet were slightly lower (~3%) than those of the control group and showed statistically significant differences. Dams receiving 3,000 or 10,000 ppm 2H4MBP in the diet displayed slight decreases in GD 6–21 body weight gain (~10%) relative to the control group that attained statistical significance (Table 2). Lower body weight gain over the GD 6–9 (10,000 ppm) and 18–21 (3,000 and 10,000 ppm) intervals, which was associated with slightly lower feed consumption over the GD 18–21 interval (Table 3), likely contributed to this response. These collective effects are minimal and would not be expected to affect normal development of the offspring. Dietary concentrations of 1,000, 3,000, and 10,000 ppm 2H4MBP resulted in average daily doses of approximately 70, 206, and 660 mg 2H4MBP/kg body weight/day during gestation, and 157, 478, and 1,609 mg/kg/day over lactation days (LD) 1–14 (Appendix H).

[b]National Toxicology Program (NTP). TR-597: Pathology tables, survival and growth curves from NTP long-term, genetic toxicology studies. Research Triangle Park, NC. 2020. https://doi.org/10.22427/NTP-DATA-TR-597

Table 2. Mean Body Weights and Body Weight Changes of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation and Lactation

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Gestation Day				
6	231.4 ± 1.9 [40]	231.1 ± 2.5 [34]	232.1 ± 1.5 [32]	231.7 ± 2.3 [36]
9	246.3 ± 2.4* [40]	248.6 ± 1.9 [34]	246.0 ± 1.4 [32]	239.7 ± 1.8* [36]
12	262.5 ± 1.6* [40]	263.2 ± 1.9 [34]	260.3 ± 1.6 [32]	258.8 ± 1.8 [36]
15	281.3 ± 1.7* [40]	282.1 ± 2.1 [34]	278.2 ± 1.8 [32]	276.4 ± 2.2 [36]
18	321.9 ± 1.8* [40]	322.1 ± 2.6 [34]	316.0 ± 2.8 [32]	314.0 ± 2.8* [36]
21	366.0 ± 2.4* [40]	364.7 ± 3.5 [34]	353.7 ± 4.9* [32]	353.8 ± 3.8* [36]
Gestation Weight Change				
6–9	14.9 ± 1.7** [40]	17.5 ± 1.3 [34]	13.9 ± 0.7 [32]	8.0 ± 1.3** [36]
9–12	16.3 ± 1.6** [40]	14.6 ± 0.6 [34]	14.3 ± 0.5 [32]	19.0 ± 0.8 [36]
12–15	18.8 ± 0.7 [40]	19.0 ± 0.5 [34]	17.9 ± 0.8 [32]	17.6 ± 0.8 [36]
15–18	40.6 ± 1.0* [40]	40.0 ± 1.0 [34]	37.8 ± 1.5 [32]	37.6 ± 1.0 [36]
18–21	44.1 ± 1.1* [40]	42.6 ± 1.4 [34]	37.6 ± 2.6* [32]	39.9 ± 1.4 [36]
6–21	134.6 ± 2.4** [40]	133.6 ± 2.9 [34]	121.6 ± 4.4** [32]	122.1 ± 3.2** [36]
Lactation Day				
1	276.3 ± 1.6** [40]	274.1 ± 2.1 [34]	269.0 ± 2.3* [32]	264.5 ± 2.2** [36]
4	289.7 ± 1.8** [40]	287.8 ± 2.3 [34]	279.8 ± 2.6** [32]	274.1 ± 2.2** [36]
7	295.9 ± 1.8 [35]	295.4 ± 2.5 [30]	289.2 ± 3.6 [27]	285.6 ± 2.5** [33]
14	312.9 ± 2.1* [35]	312.7 ± 2.7 [30]	306.9 ± 3.0 [27]	304.2 ± 3.0* [33]
21	304.8 ± 2.3* [35]	300.6 ± 2.6 [30]	295.4 ± 2.4* [27]	298.8 ± 2.7 [33]
Lactation Weight Change				
1–4	13.4 ± 1.2* [40]	13.7 ± 1.1 [34]	10.8 ± 2.0 [32]	9.6 ± 1.0 [36]
4–7	7.5 ± 1.2 [35]	6.3 ± 1.3 [30]	8.6 ± 1.5 [27]	10.3 ± 1.0 [33]
7–14	17.0 ± 1.4 [35]	17.3 ± 1.7 [30]	17.7 ± 2.5 [27]	18.6 ± 1.6 [33]
14–21	−8.1 ± 1.5 [35]	−12.0 ± 1.7 [30]	−11.6 ± 2.1 [27]	−5.4 ± 2.1 [33]
4–21	16.4 ± 1.7* [35]	11.6 ± 1.5 [30]	14.7 ± 2.3 [27]	23.5 ± 1.9* [33]

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Each dietary concentration was compared to the control with Williams' test when a trend was present ($p \leq 0.01$ from Jonckheere's trend test) or with Dunnett's test when no trend was present. Body weight data are presented in grams.

Table 3. Feed Consumption of F_0 Female Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed during Gestation and Lactation

Parameter[a]	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Gestation Day				
6–9	17.7 ± 0.5** [41]	17.9 ± 0.2 [34]	17.2 ± 0.3* [33]	13.3 ± 0.4** [36]
9–12	18.2 ± 0.2 [41]	18.6 ± 0.3 [34]	18.3 ± 0.3 [33]	19.0 ± 0.3 [36]
12–15	18.6 ± 0.2 [41]	18.6 ± 0.2 [34]	18.2 ± 0.3 [33]	18.5 ± 0.2 [36]
15–18	21.1 ± 0.2* [41]	21.3 ± 0.2 [34]	21.0 ± 0.3 [33]	20.2 ± 0.3 [36]
18–21	21.4 ± 0.2** [41]	21.3 ± 0.2 [34]	20.2 ± 0.5* [33]	19.9 ± 0.3** [36]
6–21	19.4 ± 0.2** [41]	19.6 ± 0.2 [34]	19.0 ± 0.3 [33]	18.2 ± 0.2** [36]
Lactation Day				
0–4	35.2 ± 0.6 [40]	35.0 ± 0.7 [34]	33.7 ± 1.3 [32]	37.4 ± 1.5 [35]
4–7	41.6 ± 0.6 [35]	41.4 ± 0.4 [30]	41.8 ± 1.0 [27]	41.3 ± 0.7 [33]
7–10	49.6 ± 0.8 [35]	48.4 ± 0.6 [30]	48.5 ± 1.2 [27]	47.9 ± 1.1 [33]
10–14	58.2 ± 0.8 [34]	58.4 ± 0.6 [30]	57.0 ± 1.1 [27]	55.6 ± 1.1 [33]
14–17	61.4 ± 1.1 [35]	62.6 ± 0.6 [30]	62.8 ± 0.7 [27]	59.7 ± 1.5 [33]
17–21	69.6 ± 1.1 [35]	69.4 ± 1.3 [30]	70.7 ± 1.0 [27]	69.7 ± 1.4 [33]
1–14	47.1 ± 0.5 [34]	46.9 ± 0.4 [30]	46.5 ± 0.8 [27]	46.5 ± 0.7 [32]

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Each dietary concentration was compared to the control group with Shirley's test when a trend was present or with Dunn's test when no trend was present. Feed consumption data are presented as g/animal/day.

Administration of 2H4MBP had no effects on the percentage of mated females producing pups, litter size, pup sex distribution, or numbers of male or female pups (Table 4; Table 5). The apparent decrease in the percentage of females pregnant in the 10,000 ppm group can be attributed to the seven animals that had no evidence of pregnancy as shown by the absence of implantation sites. Therefore, the lower pregnancy rate was not exposure-related given that exposure began after implantation. Dams receiving 2H4MBP did not display any adverse clinical findings before or after parturition. Litter size of the 10,000 ppm 2H4MBP group was slightly lower on PNDs 7 and 10 (Table 5).

Table 4. Summary of the Disposition of Rats during Perinatal Exposure and F_1 Allocation in the Two-year Perinatal and Postnatal Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Reproductive Performance				
Time-mated Females (GD 6)	42	35	35	43
Females Pregnant (%)[a]	41 (98%)**	34 (97%)	33 (94%)	36 (84%)
Females Not Pregnant (%)	1 (2%)	1 (3%)	2 (6%)	7 (16%)
Dams Not Delivering with Evidence of Pregnancy (%)	1[b]	0	1[c]	0
Dams with Litters on PND 0 (%)	40 (98%)	34 (100%)	32 (97%)	36 (100%)
Litters Poststandardization (PND 4)[d]	35	30	27	33
Postweaning Allocation				
F_1 Males – Chronic (Litters)[e]	50 (30)	50 (25)	50 (25)	50 (30)
F_1 Males – Interim (Litters)[f]	10 (9)	–	–	10 (8)
F_1 Females – Chronic (Litters)[e]	50 (30)	50 (25)	50 (25)	50 (30)
F_1 Females – Interim (Litters)[f]	10 (9)	–	–	10 (9)

Statistical significance for the control group indicates a significant trend test.
**Statistically significant at $p \leq 0.01$.
GD = gestation day; PND = postnatal day.
[a]Statistical analysis performed by Cochran-Armitage (trend) and Fisher Exact (pairwise) tests.
[b]Found dead on GD 23 (no adverse clinical signs) with a single fetus and no other apparent resorption sites.
[c]Undelivered (2 implantations/0 resorptions).
[d]Standardization to eight pups per litter (four pups/sex).
[e]Total number of F_1 rats included in the 105-week evaluation (number of litters from which they originated).
[f]Number of F_1 rats euthanized at the 14-week interim evaluation (number of litters from which they originated).

Table 5. Mean Number of Surviving F_1 Male and Female Rats during Lactation in the Two-year Perinatal and Postnatal Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
PND 1[a,b]				
Total	12.55 ± 0.35 [40]	12.65 ± 0.41 [34]	11.63 ± 0.65 [32]	12.53 ± 0.41 [36]
Live	12.45 ± 0.36 [40]	12.50 ± 0.41 [34]	11.44 ± 0.66 [32]	12.33 ± 0.43 [36]
% Male per Litter	51.23 ± 2.29 [40]	50.20 ± 2.34 [34]	45.84 ± 2.89 [32]	51.66 ± 2.54 [36]
% Male[c,d]	52 [498]	50 [425]	47 [366]	52 [444]
PND 4 Prestandardization	**12.43 ± 0.36 [40]**	**12.41 ± 0.41 [34]**	**11.38 ± 0.65 [32]**	**12.25 ± 0.43 [36]**
Male[a,b]				
PND 1	6.45 ± 0.35 [40]	6.29 ± 0.36 [34]	5.41 ± 0.40 [32]	6.39 ± 0.38 [36]
PND 4 Prestandardization	6.45 ± 0.35 [40]	6.24 ± 0.36 [34]	5.28 ± 0.40 [32]	6.39 ± 0.39 [36]
PND 4 Poststandardization	4.00 ± 0.00 [35]	4.00 ± 0.05 [30]	3.89 ± 0.08 [27]	4.12 ± 0.08 [33]

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Female[a,b]				
PND 1	6.00 ± 0.32 [40]	6.21 ± 0.36 [34]	6.03 ± 0.45 [32]	5.94 ± 0.36 [36]
PND 4 Prestandardization	5.98 ± 0.33 [40]	6.18 ± 0.35 [34]	6.09 ± 0.46 [32]	5.86 ± 0.34 [36]
PND 4 Poststandardization	4.00 ± 0.00 [35]	4.00 ± 0.05 [30]	4.11 ± 0.08 [27]	3.88 ± 0.08 [33]
Male and Female[a,b]				
PND 4 Poststandardization	8.00 ± 0.00 [35]	8.00 ± 0.00 [30]	8.00 ± 0.00 [27]	8.00 ± 0.00 [33]
PND 7	8.00 ± 0.00* [35]	8.00 ± 0.00 [30]	8.00 ± 0.00 [27]	7.82 ± 0.13* [33]
PND 10	8.00 ± 0.00* [35]	8.00 ± 0.00 [30]	8.00 ± 0.00 [27]	7.82 ± 0.13* [33]
PND 14	7.94 ± 0.04 [35]	7.97 ± 0.03 [30]	7.96 ± 0.04 [27]	7.82 ± 0.13 [33]
PND 17	7.94 ± 0.04 [35]	7.97 ± 0.03 [30]	7.96 ± 0.04 [27]	7.82 ± 0.13 [33]
PND 21	7.91 ± 0.05 [35]	7.97 ± 0.03 [30]	7.93 ± 0.07 [27]	7.82 ± 0.13 [33]
Survival per Litter				
Total Dead: PND 1–4[e]	5 [40]	8 [34]	8 [32]	10 [36]
Total Dead: PND 5–21[e]	3 [35]	1 [30]	2 [27]	6 [33]
Dead: PND 1–4[b,f,g]	0.125 ± 0.064 [40]	0.235 ± 0.095 [34]	0.250 ± 0.162 [32]	0.278 ± 0.102 [36]
Dead: PND 4–21[b,f,g]	0.086 ± 0.048 [35]	0.033 ± 0.033 [30]	0.074 ± 0.074 [27]	0.182 ± 0.127 [33]
Survival Ratio: PND 1–4[b,g,h]	0.998 ± 0.002 [40]	0.993 ± 0.004 [34]	0.996 ± 0.003 [32]	0.994 ± 0.005 [36]
Survival Ratio: PND 4–21[b,g,i]	0.989 ± 0.006 [35]	0.996 ± 0.004 [30]	0.991 ± 0.009 [27]	0.977 ± 0.016 [33]

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$.
PND = postnatal day.
[a]Each dietary concentration was compared to the control group with Shirley's test when a trend was present or with Dunn's test when no trend was present.
[b]Mean ± standard error [number of dams].
[c]100 × [number of live males in dietary exposure group]/[number of live males and females in dietary exposure group].
[d]No statistics done on this endpoint.
[e]Total dead in dietary concentration group [number of dams].
[f]Number dead per litter.
[g]Each dietary concentration was compared to the control group with Shirley's test when a trend is present ($p \leq 0.01$ from Jonckheere's trend test), otherwise Dunn's test is applied.
[h]Survival per litter: Number of pups prestandardization on PND 4/total live pups on PND 1.
[i]Survival per litter: Number of live pups on PND 21/number of live pups poststandardization on PND 4.

Male, female, and combined pup body weights were not significantly different across the exposure groups on PND 1. However, on PND 4, pup body weights (male, female, and both) in the 10,000 ppm exposure groups were approximately 10% lower than those of the control groups, and this response was generally observed over subsequent preweaning weights (Table 6).

Pups were weaned on PND 21, which was considered day 1 of the 2-year exposure period.

Table 6. Preweaning Pup Body Weight of Rats Following Exposure during Gestation and Lactation in the Two-year Perinatal and Postnatal Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Pup Body Weights (g)[a]				
Male				
PND 1[b,c,d]	7.32 ± 0.07 [40]	7.38 ± 0.07 [34]	7.22 ± 0.09 [31]	7.11 ± 0.08 [35]
PND 4[e,f,g]	10.65 ± 0.12** [258/40]	10.95 ± 0.12 [212/34]	10.54 ± 0.13 [169/31]	10.02 ± 0.18** [231/35]
PND 7[e,h]	16.66 ± 0.26* [140/35]	16.44 ± 0.26 [120/30]	16.29 ± 0.35 [105/27]	15.72 ± 0.28* [135/33]
PND 14[e,h]	33.72 ± 0.47** [139/35]	33.79 ± 0.38 [120/30]	32.83 ± 0.52 [104/27]	30.99 ± 0.40** [135/33]
PND 21[e,h]	53.36 ± 0.84** [136/34]	52.85 ± 0.75 [120/30]	52.61 ± 0.77 [104/27]	48.47 ± 0.74** [135/33]
Female				
PND 1[b,c,d]	6.95 ± 0.07 [40]	7.00 ± 0.08 [34]	6.86 ± 0.09 [32]	6.80 ± 0.07 [36]
PND 4[e,f,g]	10.08 ± 0.12** [239/40]	10.42 ± 0.12 [210/34]	9.94 ± 0.17 [195/32]	9.51 ± 0.17* [210/36]
PND 7[e,h]	15.56 ± 0.28* [140/35]	15.58 ± 0.26 [119/30]	15.46 ± 0.35 [111/27]	14.73 ± 0.24 [123/33]
PND 14[e,h]	32.27 ± 0.41** [138/35]	32.06 ± 0.38 [119/30]	31.37 ± 0.51 [111/27]	29.78 ± 0.33** [123/33]
PND 21[e,h]	49.49 ± 0.72** [138/35]	49.24 ± 0.79 [119/30]	49.17 ± 0.79 [110/27]	45.70 ± 0.55** [123/33]
Male and Female				
PND 1[b,c,d]	7.13 ± 0.07* [40]	7.20 ± 0.07 [34]	7.01 ± 0.08 [32]	6.95 ± 0.07 [36]
PND 4[e,f,g]	10.36 ± 0.12** [497/40]	10.70 ± 0.10 [422/34]	10.16 ± 0.16 [364/32]	9.79 ± 0.17** [441/36]
PND 7[e,h]	16.11 ± 0.26* [280/35]	16.02 ± 0.25 [239/30]	15.88 ± 0.33 [216/27]	15.23 ± 0.26 [258/33]
PND 14[e,h]	32.99 ± 0.42** [277/35]	32.94 ± 0.34 [239/30]	32.10 ± 0.49 [215/27]	30.40 ± 0.35** [258/33]
PND 21[e,h]	51.39 ± 0.73** [274/35]	51.07 ± 0.71 [239/30]	50.90 ± 0.70 [214/27]	47.21 ± 0.62** [258/33]

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
PND = postnatal day.
[a]Statistical analysis was performed using mixed models with random litter effect for both trend and pairwise tests, using the Dunnett-Hsu adjustment for multiple comparisons.
[b]Mean ± standard error [number of dams].
[c]Each dietary concentration was compared to the control group with Williams' test when a trend was present ($p \leq 0.01$ from Jonckheere's trend test) or with Dunnett's test when no trend was present.
[d]Total pup weight at PND 1 divided by number of live pups at PND 1.
[e]Mean of dam mean ± standard error [number of pups/number of dams].
[f]PND 4 prestandardization.
[g]Individual pup weights first adjusted for live litter size on PND 1.
[h]Individual pup weights first adjusted for live litter size on PND 4 poststandardization.

Postnatal Exposure

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 7 and in the Kaplan-Meier survival curves (Figure 3). Survival of all exposed groups of male and female rats was not significantly different from that of the control groups.

Table 7. Survival of Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male				
Animals Initially in Study	60	50	50	60
14-week Interim Evaluation[a]	10	0	0	10
Moribund	12	15	18	10
Natural Deaths	8	6	8	7
Animals Surviving to Study Termination	30[b]	29	24	33
Percent Probability of Survival at End of Study[c]	60	58	48	66
Mean Survival (Days)[d]	705	675	678	678
Survival Analysis[e]	p = 0.486N	p = 0.672	p = 0.203	p = 0.706N
Female				
Animals Initially in Study	60	50	50	60
14-week Interim Evaluation[a]	10	0	0	10
Moribund	15	13	10	16
Natural Deaths	5	4	6	8
Animals Surviving to Study Termination	30	33	34	26
Percent Probability of Survival at End of Study[c]	60	66	68	52
Mean Survival (Days)[d]	659	682	692	658
Survival Analysis[e]	p = 0.231	p = 0.385N	p = 0.238N	p = 0.603

[a]Excluded from survival analysis.
[b]Includes one animal that died during the last week of the study.
[c]Kaplan-Meier determinations.
[d]Mean of litter means of all deaths (uncensored, censored, and study termination).
[e]The result of the Cox proportional hazards trend test[67] is in the control column, and the results of the proportional hazards pairwise comparisons with the control groups are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by N.

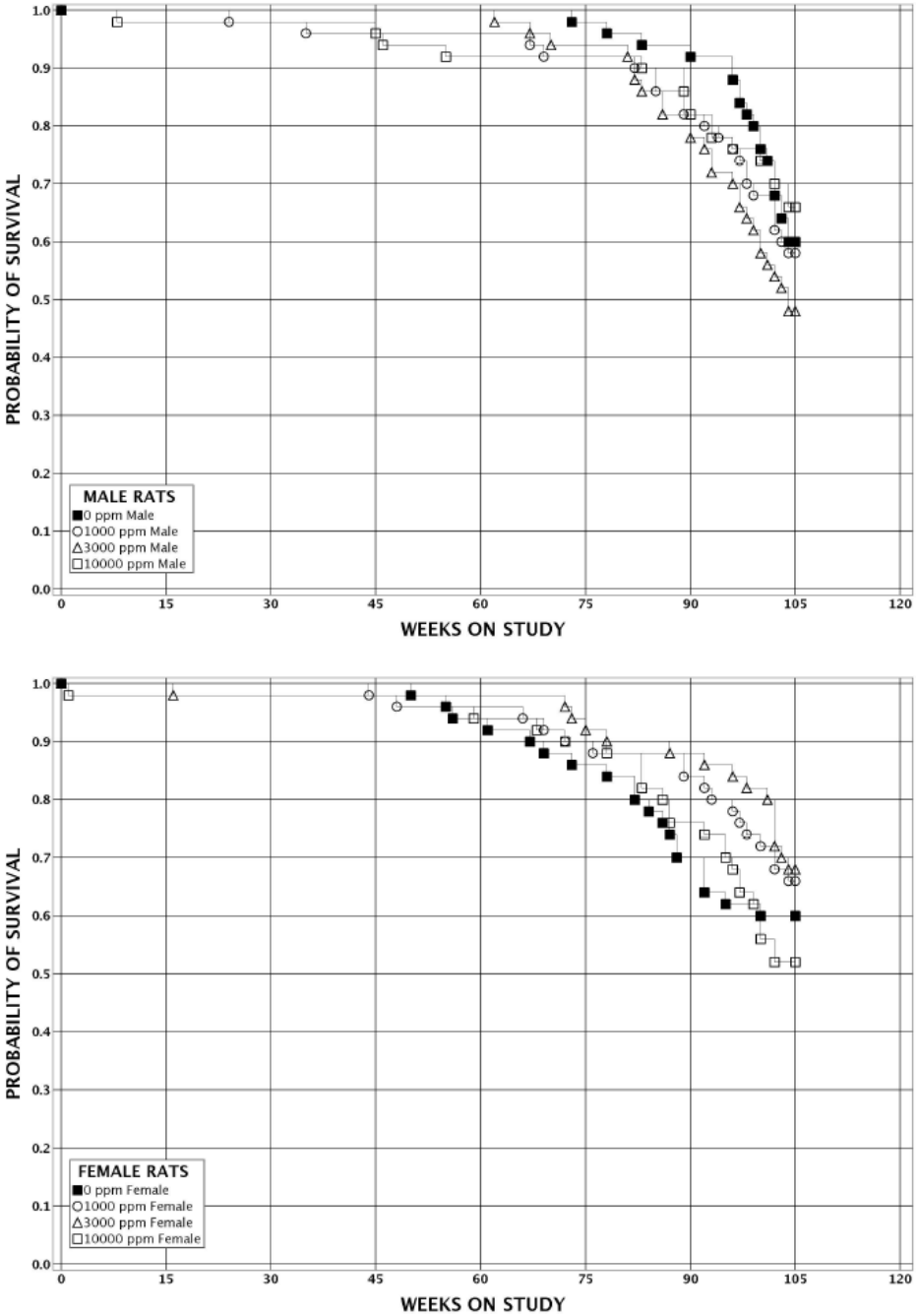


Figure 3. Kaplan-Meier Survival Curves for Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed for Two Years

Fourteen-week Interim Evaluation

Groups of 10 male and 10 female rats were exposed to 0 or 10,000 ppm 2H4MBP for 14 weeks. At the 14-week interim evaluation, the mean body weight of the 10,000 ppm males was not significantly different from that of the control males, but the mean body weight of the 10,000 ppm females was significantly decreased and was approximately 87% that of the control group (Table 8). In males, the absolute and relative liver and right kidney weights were increased in the 10,000 ppm group compared to the control group. In females, the absolute kidney weight was significantly decreased, and the relative liver weight was significantly increased relative to the control group (Table 8).

The incidence of mixed-cell cellular infiltration in the liver was significantly increased in 10,000 ppm males relative to the control group (Table 8; Appendix H). These cellular infiltrates were composed of mononuclear cells with scarce neutrophils and had no specific predisposition to a specific area of the liver lobule. It is unlikely that the cellular infiltrates, which were all of minimal severity, would be responsible for the changes in the liver weights observed in male rats at this time point (Table 8; Appendix H). No other histologic findings were observed to explain the differences in organ weights, but in the females, body weight changes could have influenced the absolute kidney weight decrease and the relative liver weight increase. However, the increase in relative liver weight in exposed females was accompanied by a nonsignificant absolute liver weight increase, so it is unlikely that body weight was responsible for the liver weight changes (Table 8).

As a part of the 14-week interim evaluation in the F_1 rat study, transcriptome analysis was performed on RNA extracted from microarray study male rat livers from the 10,000 ppm and control groups. The observed effects on transcription were consistent with a mild induction of xenobiotic metabolism-related processes that is likely related to the observed liver weight increases. Analysis of a subset of estrogen-responsive genes showed no change in response to 2H4MBP (Appendix E).

Table 8. Select Organ Weights, Organ-Weight-to-Body-Weight Ratios, and Histological Findings in Rats at the 14-week Interim of the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	10,000 ppm
n	10	10
Male		
Necropsy Body Wt. (g)[a]	394.6 ± 7.9	386.8 ± 6.8
R. Kidney[a]		
Absolute (g)	1.18 ± 0.03**	1.37 ± 0.03**
Relative (mg/g)[b]	3.00 ± 0.06**	3.54 ± 0.07**
Liver[a]		
Absolute (g)	13.94 ± 0.49**	16.41 ± 0.46**
Relative (mg/g)	35.25 ± 0.68**	42.38 ± 0.75**
Histological Findings[c]		
Liver[d]	10	10
Infiltration cellular, mixed cell[e]	2 (1.0)[f]	8** (1.0)
Female		
Necropsy Body Wt. (g)	261.7 ± 6.3**	228.7 ± 4.9**
R. Kidney		
Absolute (g)	0.79 ± 0.01*	0.71 ± 0.02**
Relative (mg/g)	3.01 ± 0.04	3.09 ± 0.08
Liver		
Absolute (g)	8.77 ± 0.38	9.64 ± 0.28
Relative (mg/g)	33.43 ± 0.91**	42.15 ± 0.68**
Histological Findings		
Liver	(10)	(10)
Infiltration cellular, mixed cell	2 (1.0)	3 (1.0)

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are displayed as mean ± SEM (N). Statistical analysis performed by Jonckheere's (trend) and Williams' or Dunnett's (pairwise) tests.
[b]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.
[c]Statistical analysis for histological findings performed using the Poly-3 test.
[d]Number of animals examined microscopically.
[e]Number of animals with lesion.
[f]Average severity grade of lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

Chronic Exposure

Body Weights, Feed and Compound Consumption, and Clinical Observations

Male rats exposed to 10,000 ppm 2H4MBP displayed consistently lower mean body weights (~10%) after week 69 relative to the control group and were 20% lower than the control group mean at study end (Table 9; Figure 4). Females exposed to 10,000 ppm 2H4MBP displayed a similar response (~10% lower than the control group mean after week 17), but after week 45 this group displayed mean body weights that were approximately 16% lower than the control group, and mean body weights were 24% lower than the control group mean at study end. Mean body weights of females exposed to 3,000 ppm 2H4MBP were lower than the control group mean after week 77 (~10%) (Table 10).

Feed consumption by 2H4MBP-exposed male and female rats was not significantly different from that by the control groups throughout the study (Appendix H). Dietary concentrations of 1,000, 3,000, and 10,000 ppm resulted in average daily doses of approximately 58, 168, and 585 mg 2H4MBP/kg body weight for males and 60, 180, and 632 mg/kg for females. No clinical findings in exposed groups of male or female rats were considered to be related to 2H4MBP exposure (Appendix H).

Table 9. Mean Body Weights and Survival of Male Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm		1,000 ppm			3,000 ppm			10,000 ppm		
Day	**Av. Wt. (g)**	**No. of Litters**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Litters**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Litters**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Litters**
1	56.2	30	56.2	100	25	56.0	100	25	51.1	91	30
8	85.5	30	85.1	100	25	84.2	99	25	74.5	87	30
15	130.9	30	127.4	97	25	125.2	96	25	109.6	84	30
22	177.3	30	173.5	98	25	170.8	96	25	151.7	86	30
29	224.2	30	218.2	97	25	217.2	97	25	194.5	87	30
36	270.4	30	262.5	97	25	260.8	96	25	236.9	88	30
43	304.2	30	295.3	97	25	297.4	98	25	272.0	89	30
50	332.6	30	324.4	98	25	324.6	98	25	299.4	90	30
57	350.4	30	342.7	98	25	343.4	98	25	321.3	92	30
64	368.3	30	361.0	98	25	358.8	97	25	337.4	92	30
71	382.9	30	372.8	97	25	370.9	97	25	351.7	92	30
78	395.7	30	387.2	98	25	384.1	97	25	364.4	92	30
85	407.4	30	398.2	98	25	394.7	97	25	374.1	92	30
92	416.6	30	408.0	98	25	404.1	97	25	383.1	92	30
120	443.8	29[a]	429.8	97	25	428.4	97	25	401.2	90	28[a]
148	469.2	29	447.8	95	25	453.6	97	25	423.9	90	28
176	482.1	29	467.6	97	25	467.9	97	25	439.1	91	28
204	502.2	29	490.0	98	25	481.8	96	25	458.2	91	28
232	511.4	29	495.2	97	25	496.0	97	25	462.8	91	28
260	522.1	29	512.2	98	25	509.5	98	25	479.5	92	28
288	538.5	29	524.4	97	25	518.9	97	25	489.0	91	28
316	548.5	29	533.2	97	25	529.2	97	25	495.1	90	28
344	555.0	29	538.3	97	25	541.8	98	25	502.8	91	28
372	570.2	29	551.9	97	25	557.0	98	25	521.0	91	28
400	579.2	29	561.6	97	25	563.0	97	25	526.5	91	28
428	585.1	29	557.5	96	25	576.1	99	25	531.8	91	28
456	594.5	29	570.4	96	25	587.9	99	25	536.8	90	28
484	606.6	29	575.2	95	24	592.7	98	25	543.1	90	28
512	610.7	29	581.6	95	24	598.1	98	25	546.2	89	28
540	608.6	29	573.9	94	24	598.7	98	25	543.0	89	28
568	613.1	29	577.9	94	24	597.7	98	25	537.9	88	28
596	617.6	29	590.5	96	24	599.1	97	23	538.6	87	27
624	613.2	29	584.4	95	23	594.6	97	23	533.1	87	27
652	601.5	28	584.7	97	23	592.9	99	22	528.6	88	25
680	590.3	26	572.0	97	23	590.8	100	21	510.1	86	25
708	592.2	23	547.4	92	20	584.5	99	21	486.7	82	24
EOS	**603.4**	**21**	**556.3**	**92**	**19**	**575.9**	**96**	**18**	**480.3**	**80**	**23**

EOS = end of study.
[a]Fourteen-week interim necropsy occurred between this day and the previous scheduled weigh day.

Table 10. Mean Body Weights and Survival of Female Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

Day	0 ppm		1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters
1	53.7	30	54.5	102	25	55.1	103	25	49.4	92	30
8	80.5	30	81.0	101	25	80.5	100	25	71.6	89	30
15	115.3	30	114.5	99	25	114.0	99	25	100.5	87	30
22	145.7	30	145.0	100	25	143.0	98	25	128.8	88	30
29	162.6	30	165.3	102	25	162.8	100	25	151.0	93	30
36	182.3	30	183.9	101	25	180.3	99	25	168.8	93	30
43	199.9	30	198.5	99	25	195.0	98	25	181.6	91	30
50	212.7	30	212.0	100	25	209.1	98	25	193.6	91	30
57	221.0	30	223.3	101	25	216.7	98	25	200.4	91	30
64	229.7	30	229.8	100	25	225.1	98	25	207.8	91	30
71	235.0	30	236.2	101	25	230.7	98	25	213.5	91	30
78	240.2	30	239.9	100	25	234.4	98	25	218.0	91	30
85	246.0	30	246.9	100	25	238.8	97	25	223.5	91	30
92	251.2	30	250.5	100	25	243.8	97	25	227.9	91	30
120	266.0	29[a]	262.2	99	25	254.3	97	25	237.9	89	29[a]
148	276.1	29	275.0	100	25	269.3	98	25	245.8	89	29
176	282.7	29	280.4	99	25	273.8	97	25	252.4	89	29
204	293.3	29	283.6	97	25	278.9	95	25	257.4	88	29
232	300.6	29	291.0	97	25	286.7	95	25	260.8	87	29
260	306.7	29	296.6	97	25	291.3	95	25	264.7	86	29
288	311.5	29	300.5	97	25	294.2	94	25	267.6	86	29
316	316.7	29	303.0	96	25	295.8	93	25	267.0	84	29
344	323.2	29	307.9	95	25	301.5	93	25	269.9	84	29
372	333.3	29	312.4	94	25	305.7	92	25	275.6	83	29
400	342.0	29	318.9	93	25	310.4	91	25	279.1	82	28
428	339.2	28	321.5	95	25	313.9	93	25	281.7	83	28
456	348.1	28	326.9	94	25	318.4	92	25	286.5	82	28
484	355.6	28	329.0	93	25	322.6	91	25	286.6	81	27
512	358.6	28	332.4	93	25	325.6	91	25	287.8	80	27
540	368.4	28	334.0	91	24	327.0	89	25	292.6	79	27
568	373.4	27	341.0	91	24	331.0	89	25	297.5	80	27
596	378.2	27	348.0	92	24	333.6	88	25	295.9	78	27
624	386.2	27	351.0	91	24	336.6	87	25	303.1	79	26
652	380.1	24	352.0	93	24	330.2	87	25	311.1	82	26
680	386.1	23	359.0	93	23	335.8	87	25	307.0	80	24
708	384.0	23	371.1	97	23	335.9	88	25	306.9	80	21
EOS	**401.4**	**23**	**367.1**	**92**	**22**	**346.0**	**87**	**25**	**303.7**	**76**	**19**

EOS = end of study.
[a]Fourteen-week interim necropsy occurred between this day and the previous scheduled weigh day.

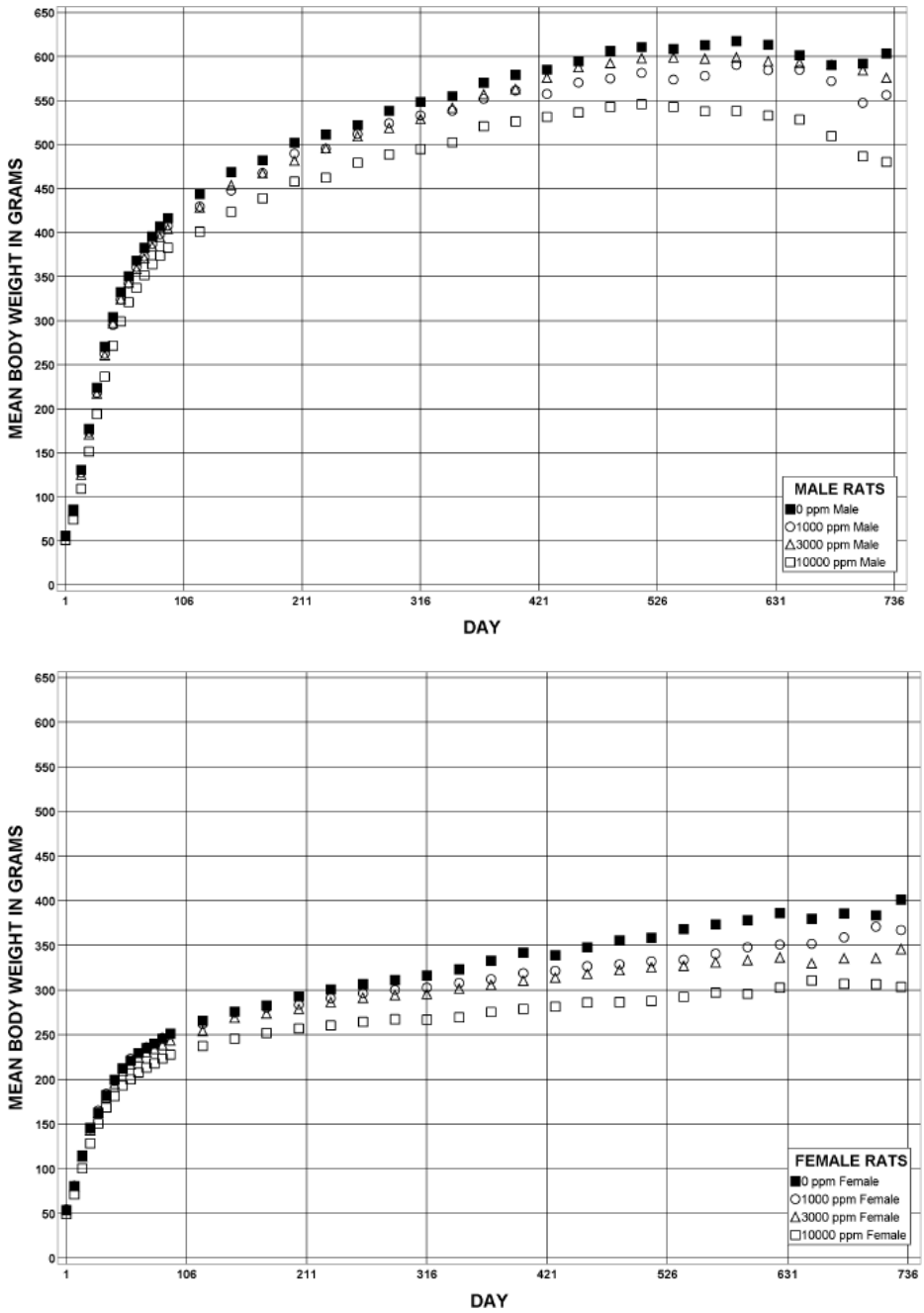


Figure 4. Growth Curves for Rats Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed for Two Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidence of neoplasms and nonneoplastic lesions of the brain and spinal cord, thyroid gland, uterus, adrenal cortex, testis, pancreas, and mammary gland. Summaries of the incidence of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms are presented in Appendix H.

Brain: Malignant meningiomas occurred only in treated animals, with incidences in males at the end of the 2-year study of 0/50, 1/50, 3/50, and 0/50 (Table 11; Appendix H). In addition, there was one malignant meningioma in the spinal cord of a 3,000 ppm animal (which did not have a meningioma in the brain). The spinal cord was examined only when an animal displayed clinical neurological signs, so the actual incidence of meningiomas in the spinal cord may be underrepresented. In females, there was one malignant meningioma (in the brain of a 3,000 ppm animal).

Malignant meningiomas in the brain varied somewhat in appearance, both between neoplasms and within a single neoplasm. All of the neoplasms were poorly delineated, cellular neoplasms that were either invading into the brain parenchyma directly, or following along the meninges and vessels deep into the brain (Figure 5 and Figure 6). One neoplasm also involved the pituitary gland, and another involved the trigeminal ganglion. Within a single neoplasm, there was some variability in cell size and shape, with some of the neoplasms displaying more anisocytosis and pleomorphism than others. In some areas, neoplasms had a more sarcomatous appearance, consisting of interlacing bundles of spindle-shaped cells with indistinct cell borders. Cells in these areas tended to have indistinct cell borders, eosinophilic fibrillary cytoplasm, and oval vesicular nuclei. Other areas were characterized by sheets of cells, with round, open-faced nuclei and vacuolated cytoplasm (Figure 5 and Figure 6). In still other areas, the cells were arranged in whorls; these areas tended to have elongated nuclei and indistinct cell borders. Within some neoplasms, there were focal areas of hemorrhage and coagulative necrosis. Mitotic figures were not common in most areas of the lesions, but 1–2 per high power field could be found in some sections of a couple of the tumors. In the one case involving the spinal cord, the neoplasm appeared to be based within the meninges, with infiltration into the underlying spinal cord and spinal nerves. The neoplastic cells within the meninges appeared more epithelial than sarcomatous, although both types were present.

Table 11. Incidences of Neoplasms of the Brain in Male Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
n[a]	50	50	50	50
Meningioma, Malignant[b]				
Overall rate[c]	0/50 (0%)	1/50 (2%)	3/50 (6%)	0/50 (0%)
Rate per litters[d]	0/29 (0%)	1/25 (4%)	2/25 (8%)	0/28 (0%)
Adjusted rate[e]	0%	2.3%	7%	0%
Terminal rate[f]	0/30 (0%)	0/29 (0%)	1/24 (4%)	0/33 (0%)
First incidence (days)	–	162	430	–
Rao-Scott-adjusted Poly-3 test[g]	p = 0.583N	p = 0.706	p = 0.291	(e)

(e) = value of statistic could not be computed.
[a]Number of animals with tissue examined microscopically.
[b]Historical control incidence for all routes of 2-year studies: 0/340.
[c]Number of animals with neoplasm per number of animals necropsied.
[d]Number of litters with neoplasm-bearing animals per number of litters examined at site.
[e]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[f]Observed incidence at study termination.
[g]Beneath the control incidence is the p value associated with the trend test. Beneath the exposed group incidence are the p values corresponding to pairwise comparisons between the control group and that exposed group. The Rao-Scott test adjusts the Poly-3 test (which accounts for differential mortality in animals that do not reach terminal euthanasia) for within-litter correlation. A negative trend or a lower incidence in an exposure group is indicated by N.

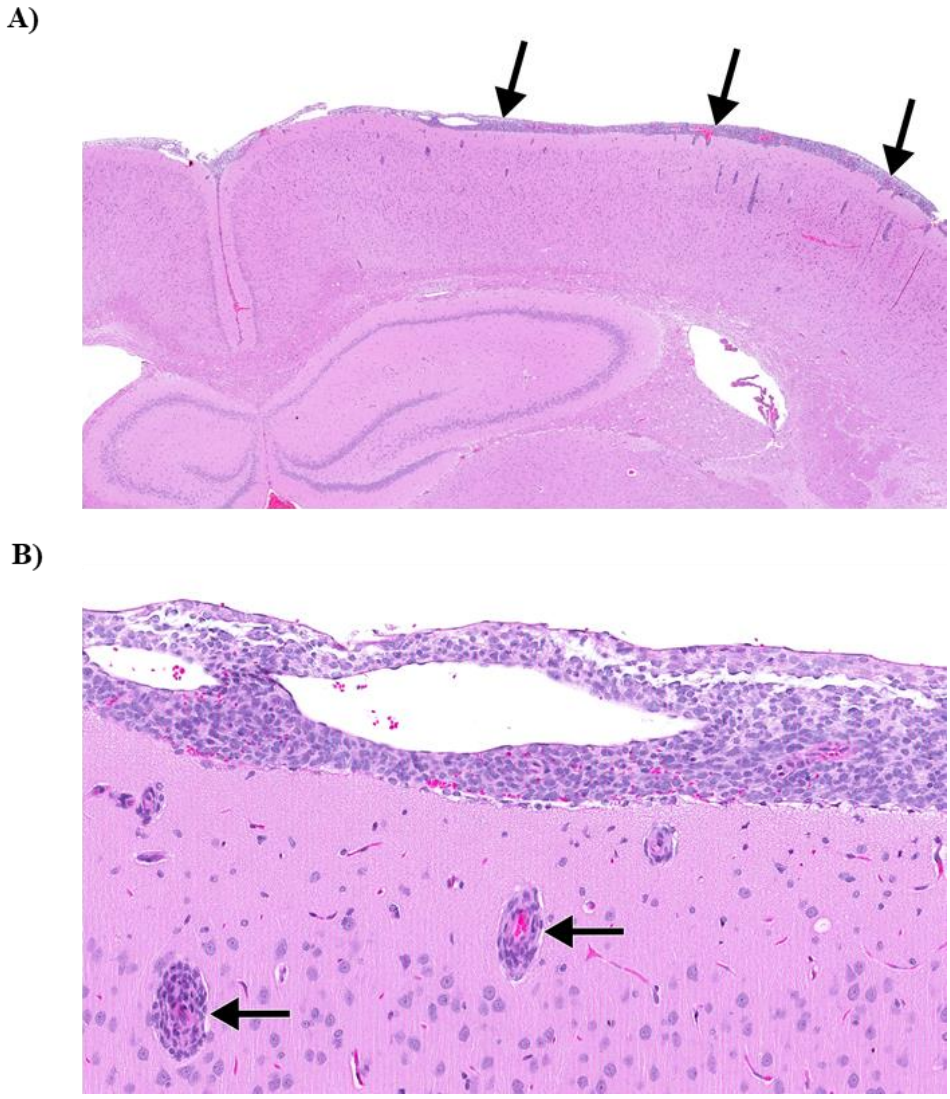
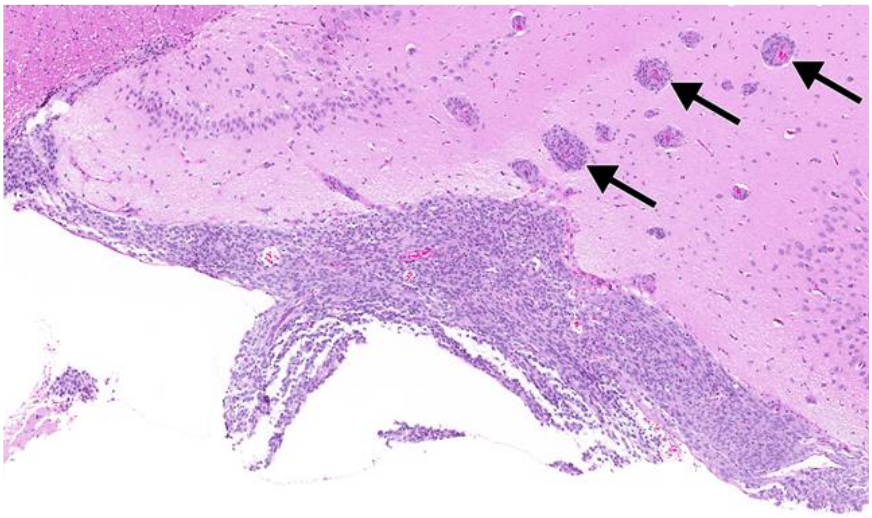


Figure 5. Malignant Meningioma in the Brain of a Male Sprague Dawley Rat Exposed to 1,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

This animal was humanely euthanized on study day 162. A) Widespread thickening of the meninges can be observed and is due to a highly cellular neoplasm (arrows). B) Higher magnification of panel A shows closely packed cells with oval nuclei and indistinct borders. The cells contain wispy, lightly eosinophilic cytoplasm and surround small vessels within the brain (arrows).

A)



B)

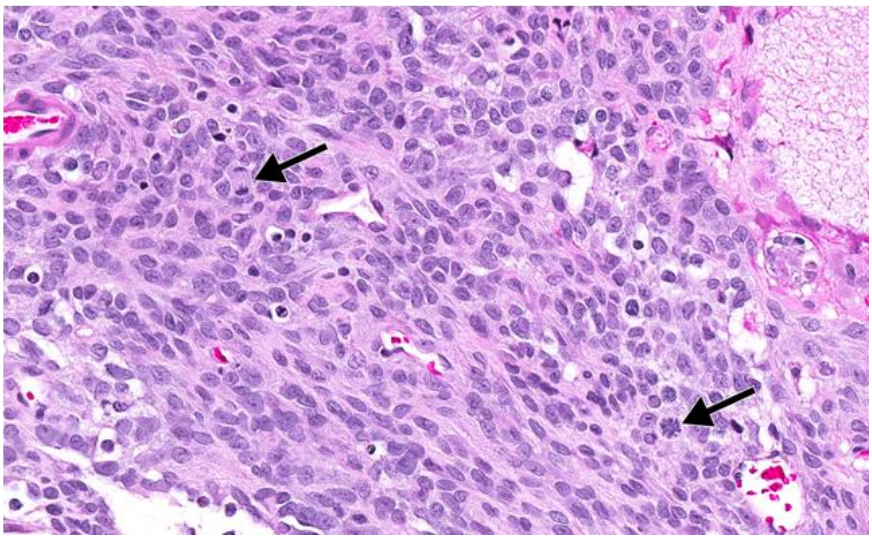


Figure 6. Malignant Meningioma in the Brain of a Male Sprague Dawley Rat Exposed to 3,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) Another example of a malignant meningioma, this one is from a male Sprague Dawley rat exposed to 3,000 ppm 2H4MBP. As in Figure 5, within the meninges is a dense proliferation of cells that can be seen surrounding blood vessels deep within the brain (arrows). B) A higher magnification of the neoplasm in panel A shows mitotic figures (arrows) among the neoplastic cells.

Thyroid gland: The incidence of C-cell adenomas in 3,000 ppm females was significantly greater than that in the control group at the end of the 2-year study (Table 12; Appendix H). Only one female rat, in the 10,000 ppm group, had bilateral C-cell adenomas; the rest were unilateral lesions. One animal (in the 1,000 ppm group) had both a C-cell adenoma and a C-cell carcinoma (in the opposite gland) but otherwise, all C-cell lesions occurred in unique animals. The incidences of C-cell carcinomas and hyperplasia in exposed groups of female rats were similar to those in the control group. There was no significant exposure concentration-related difference in the incidence of C-cell adenomas in male rats (0 ppm, 7/50; 1,000 ppm, 10/50; 3,000 ppm, 8/50; 10,000 ppm 8/50) when compared to the control group. C-cell adenomas were discrete masses of C-cells, typically larger than five thyroid gland follicles in diameter (Figure 7). The neoplasms caused some compression of adjacent follicles, but did not invade the thyroid gland capsule, and were composed of round-to-oval cells with pale cytoplasm and round nuclei. The cells were arranged in nests and clusters that were separated by a delicate fibrovascular stroma (Figure 7).

Table 12. Incidences of Neoplastic and Nonneoplastic Lesions of the Thyroid Gland in Female Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
n[a]	50	50	50	50
C-cell, Hyperplasia[b]	11 (2.0)c	11 (2.4)	9 (1.9)	9 (2.4)
C-cell, Adenoma[d]				
Overall rate[e]	5/50 (10%)	11/50 (22%)	17/50 (34%)	10/50 (20%)
Rate per litters[f]	5/29 (17%)	10/25 (40%)	15/25 (60%)	9/29 (31%)
Adjusted rate[g]	12.4%	25.1%	37.2%	24.3%
Terminal rate[h]	3/30 (10%)	9/33 (27%)	12/34 (35%)	6/26 (23%)
First incidence (days)	582	529	540	581
Rao-Scott-adjusted Poly-3 test[i]	p = 0.326	p = 0.108	p = 0.008	p = 0.128
C-cell, Carcinoma[j]				
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)	1/50 (2%)
Rate per litter	1/29 (3%)	1/25 (4%)	0/25 (0%)	1/29 (3%)
Adjusted rate	2.6%	2.3%	0%	2.5%
Terminal rate	1/30 (3%)	1/33 (3%)	0/34 (0%)	1/26 (4%)
First incidence (days)	730 (T)	730 (T)	–[k]	730 (T)
Rao-Scott-adjusted Poly-3 test	(n)	(n)	(n)	(n)

(T) = terminal euthanasia; (n) = no statistical test run.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[d]Historical control incidence for all routes of 2-year studies (mean ± standard deviation): 38/339 (11.85% ± 7.01%); range: 4% to 22%.
[e]Number of animals with neoplasm per number of animals necropsied.
[f]Number of litters with neoplasm-bearing animals per number of litters examined at site.
[g]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[h]Observed incidence at study termination.
[i]Beneath the control incidence is the p value associated with the trend test. Beneath the exposed group incidence are the p values corresponding to pairwise comparisons between the control group and that exposed group. The Rao-Scott test adjusts the Poly-3 test (which accounts for differential mortality in animals that do not reach terminal euthanasia) for within-litter correlation. A negative trend or a lower incidence in an exposure group is indicated by N.
[j]Historical control incidence: 4/339 (1.33% ± 1.63%); range: 0% to 4%.
[k]Not applicable; no neoplasms in animal group.

A) **B)**

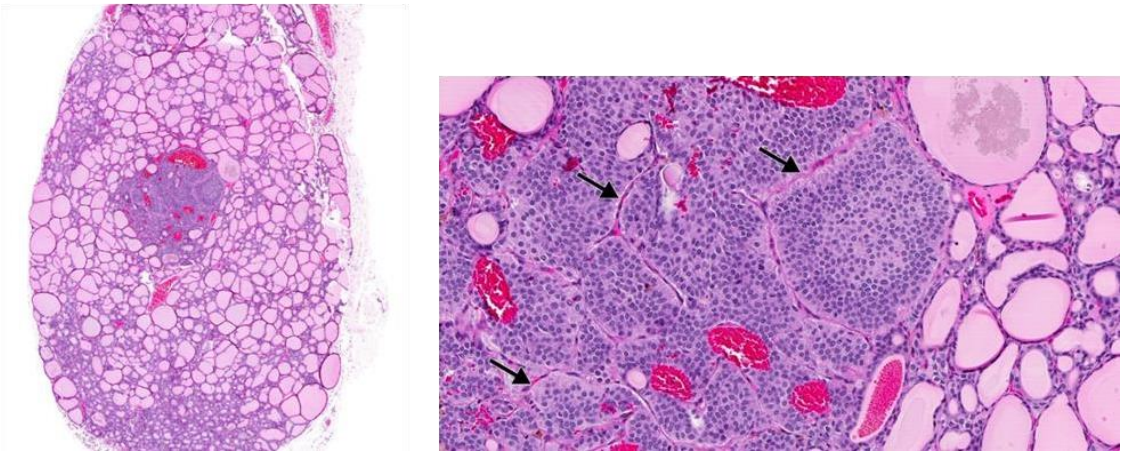


Figure 7. C-cell Adenoma in the Thyroid Gland of a Female Sprague Dawley Rat Exposed to 3,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) The C-cell adenoma is a discrete mass of several nests, or clusters, of cells separated by a delicate fibrovascular stroma. B) Higher magnification of the C-cell adenoma; at this magnification, the fibrovascular stroma that separates the nests of C-cells is more apparent (arrows). The cells that make up the adenoma have round nuclei and pale staining cytoplasm.

Uterus: At 2 years, a significantly increased incidence of stromal polyp occurred in 3,000 ppm females when compared to the control group (Table 13; Appendix H). Incidences of stromal sarcoma, which can occasionally arise within stromal polyps,[103] were not significantly increased relative to the control group in exposed females, and only occurred in the 1,000 and 3,000 ppm groups (Table 13; Appendix H). Stromal polyps typically were sessile or pedunculated masses of loosely organized stromal cells observed within the uterine lumen (Figure 8). Blood vessels and variable numbers of glands could be found within the polyps, which were covered by a single layer of cuboidal or columnar epithelium. A few polyps were partially lined by squamous epithelium; this was observed when the polyp arose in areas of moderate to marked squamous metaplasia of the uterus. Stromal sarcomas were poorly demarcated, highly cellular neoplasms composed of pleomorphic spindle cells with indistinct borders (Figure 9). If a stromal sarcoma arises within a stromal polyp, only the stromal sarcoma is diagnosed. In the two cases in which both were diagnosed in the same rat, the polyp and the sarcoma were considered separate, unique neoplasms.

Compared to the control group, there was a significantly increased incidence of atypical endometrium hyperplasia of the uterus in 3,000 ppm females (Table 13; Appendix H). Atypical hyperplasia is considered a preneoplastic lesion of the uterine epithelium,[51] but 3,000 ppm females had a significantly decreased incidence of adenocarcinoma of the uterus (Table 13; Appendix H). Atypical hyperplasia involved both the endometrial glands and the surface epithelium (Figure 10). Affected endometrial glands were characterized by clusters of enlarged glands lined by disorganized, stratified, large epithelial cells displaying pleomorphism and anisokaryosis; thickened papillary projections and infoldings often contained clear vacuoles within the cells or spaces reminiscent of glandular lumen formation (Figure 10). Atypical hyperplasia affecting the surface epithelium consisted of branching, frond-like projections of epithelial cells on a fibrovascular stalk extending into the uterine lumen.

Table 13. Incidences of Neoplastic and Nonneoplastic Lesions of the Uterus in Female Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
n[a]	50	50	50	50
Endometrium, Atypical Hyperplasia[b]	9 (2.0)[c]	14 (1.5)	19* (1.4)	14 (2.1)
Adenocarcinoma[d]				
Overall rate[e]	5/50 (10%)	3/50 (6%)	0/50 (0%)	4/50 (8%)
Rate per litters[f]	5/29 (17%)	3/25 (12%)	0/25 (0%)	4/29 (14%)
Adjusted rate[g]	12.6%	7.0%	0.0%	9.9%
Terminal rate[h]	4/30 (13%)	3/33 (9%)	0/34 (0%)	4/26 (15%)
First incidence (days)	644	730 (T)	–[i]	730 (T)
Rao-Scott-adjusted Poly-3 test[j]	p = 0.555	p = 0.325N	p = 0.036N	p = 0.486N
Stromal Polyp, Multiple	1	3	3	0
Stromal Polyp (includes multiple)[k]				
Overall rate	8/50 (16%)	15/50 (30%)	18/50 (36%)	10/50 (20%)
Rate per litters	8/29 (28%)	12/25 (48%)	16/25 (64%)	10/29 (34%)
Adjusted rate	20.4%	33.9%	39.9%	24.4%
Terminal rate	8/30 (27%)	12/33 (36%)	14/34 (41%)	7/26 (27%)
First incidence (days)	730 (T)	529	681	599
Rao-Scott-adjusted Poly-3 test	p = 0.415N	p = 0.132	p = 0.049	p = 0.424
Stromal Sarcoma	0	1	2	0
Stromal Polyp or Stromal Sarcoma (Combined)[l]				
Overall rate	8/50 (16%)	15/50 (30%)	19/50 (38%)	10/50 (20%)
Rate per litters	8/29 (28%)	12/25 (48%)	17/25 (68%)	10/29 (34%)
Adjusted rate	20.4%	33.9%	41.5%	24.4%
Terminal rate	8/30 (27%)	12/33 (36%)	14/34 (41%)	7/26 (27%)
First incidence (days)	730 (T)	529	525	599
Rao-Scott-adjusted Poly-3 test	p = 0.409N	p = 0.132	p = 0.035	p = 0.424

*Significantly different ($p \leq 0.05$) from the control group by the Rao-Scott-adjusted Poly-3 test.
(T) = terminal euthanasia.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[d]Historical control incidence for all routes of 2-year studies (mean ± standard deviation): 11/150 (7.33% ± 4.62%); range: 2% to 10%.
[e]Number of animals with neoplasm per number of animals necropsied.
[f]Number of litters with neoplasm-bearing animals per number of litters examined at site.
[g]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.
[h]Observed incidence at terminal euthanasia.
[i]Not applicable; no neoplasms in animal group.
[j]Beneath the control incidence is the p value associated with the trend test. Beneath the exposed group incidence are the p values corresponding to pairwise comparisons between the control group and that exposed group. The Rao-Scott test adjusts the Poly-3 test (which accounts for differential mortality in animals that do not reach terminal euthanasia) for within-litter correlation. A negative trend or a lower incidence in an exposure group is indicated by N.
[k]Historical control incidence: 34/150 (22.67% ± 8.33%); range: 16% to 32%.
[l]Historical control incidence: 36/150 (24% ± 8%); range: 16% to 32%.

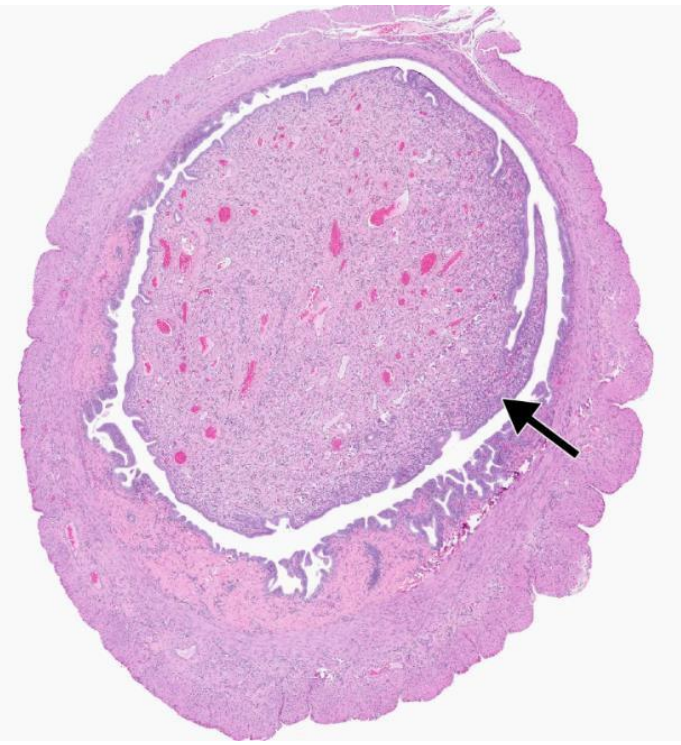
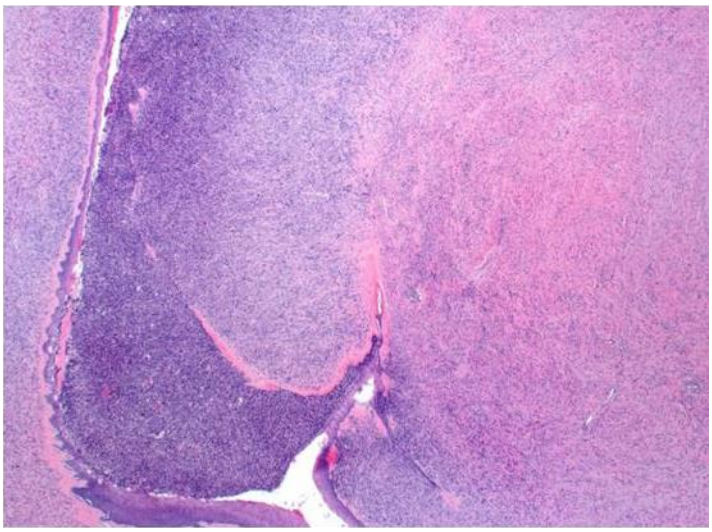


Figure 8. Stromal Polyp in the Uterus of a Female Sprague Dawley Rat Exposed to 3,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A cross section of a stromal polyp is visible within the lumen of the uterus. This polyp (arrow) is primarily composed of connective tissue with few glands; some polyps contain more glands.

A)



B)

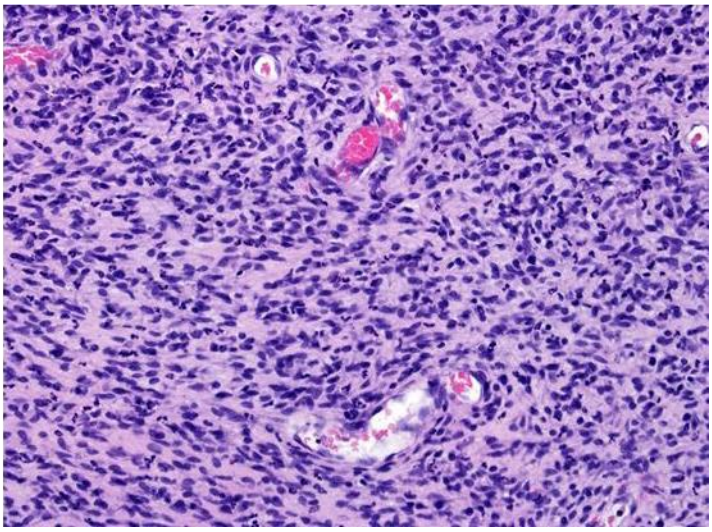


Figure 9. Stromal Sarcoma in the Uterus of Female Sprague Dawley Rat Exposed to 1,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) The sarcoma is a poorly demarcated, highly cellular mass. B) Magnification of panel A. A dense population of neoplastic cells is evident with oval to elongated nuclei and indistinct cell borders.

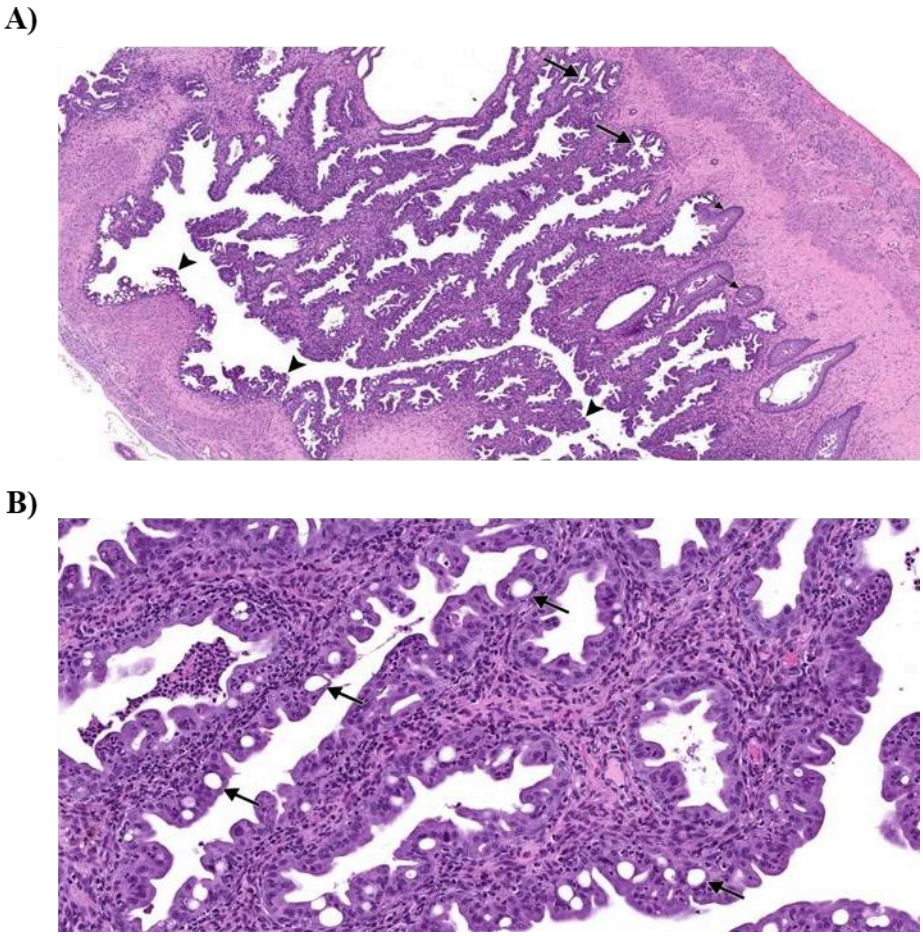


Figure 10. Atypical Hyperplasia of the Uterus in a Female Sprague Dawley Rat Exposed to 3,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) Atypical hyperplasia affected both the luminal surface epithelium (arrowheads) and the glandular epithelium (large arrows). This animal also had squamous metaplasia of the epithelium in some glands (small arrows). B) Higher magnification of hyperplasia imaged in panel A; glands are lined by enlarged, stratified, disorganized, and pleomorphic cells that frequently contain large vacuoles (arrows).

Adrenal cortex: The incidences of focal hypertrophy were significantly increased in 1,000 and 3,000 ppm females compared to the control group at the end of the 2-year study (Table 14; Appendix H). Hypertrophy in the cortex was characterized by a focal area of enlarged cells, without a concomitant increase in cell numbers. Affected cells had increased amounts of pale to brightly eosinophilic cytoplasm. Minimal to mild lesions often involved the zona glomerulosa with extension into the zona fasciculata, although some were observed only in the zona fasciculata (Figure 11). Larger lesions seemed to involve the entire thickness of the cortex, and the cells often displayed cytoplasmic vacuolation (Figure 11). One control female had bilateral lesions; all others were unilateral.

Table 14. Incidences of Nonneoplastic Lesions of the Testes, Pancreas, and Adrenal Cortex of Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male[a]	50	50	50	50
Testes				
Arteriole, necrosis, fibrinoid[b]	16* (2.3)[c]	19 (2.6)	16 (2.6)	25* (2.4)
Interstitial cell, hyperplasia	1* (1.0)	0	0	5 (2.0)
Pancreas				
Arteriole, inflammation, chronic active	4 (1.5)	15* (1.2)	10 (1.4)	11 (1.3)
Female[a]	50	50	50	50
Adrenal Cortex				
Hypertrophy, focal	24 (2.0)	42** (1.8)	39* (1.6)	27 (1.7)

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control indicates a significant trend test.
*Statistically significant at $p \leq 0.05$ by the Rao-Scott test; **$p \leq 0.01$.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked).

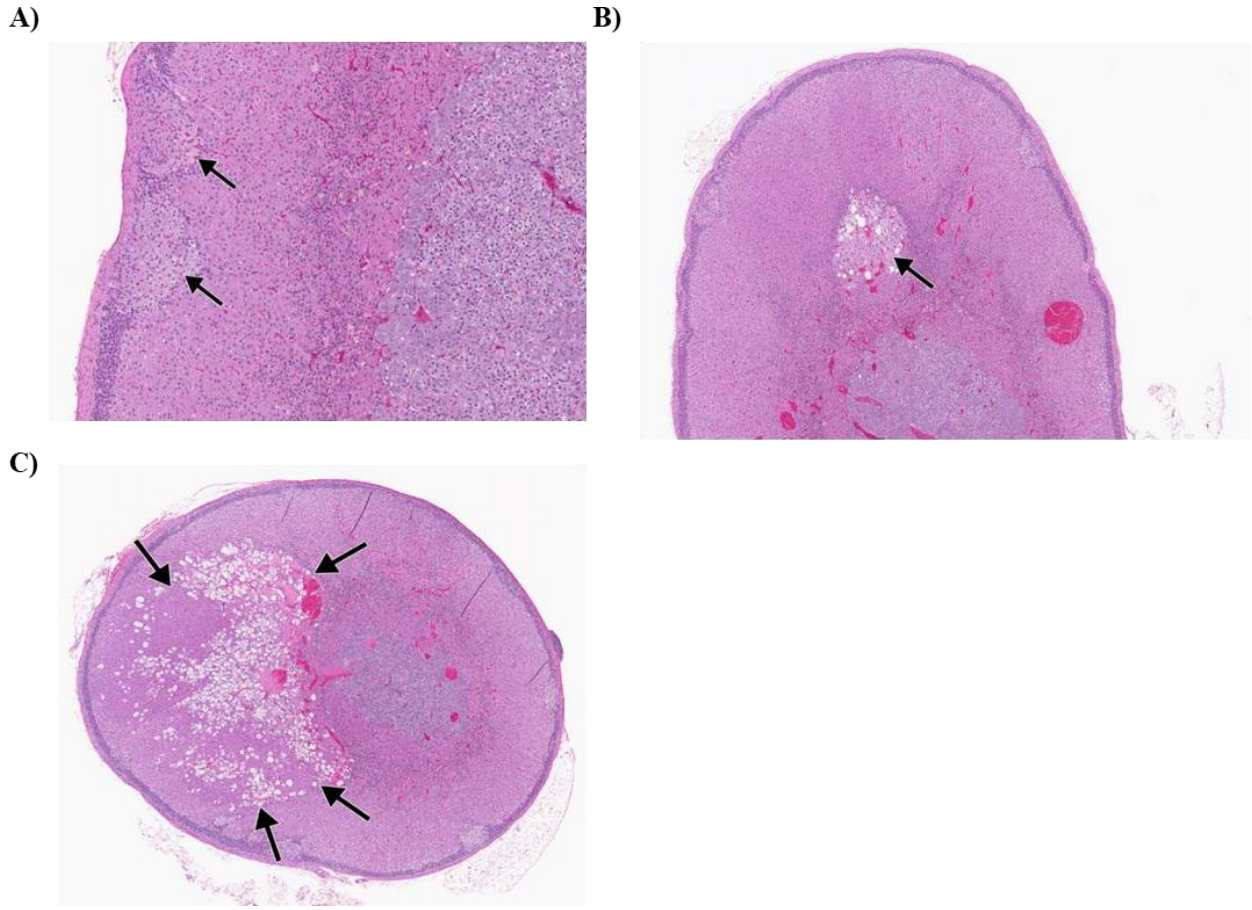


Figure 11. Focal Hypertrophy in the Adrenal Cortex from a Female Sprague Dawley Rat Exposed to 3,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) Minimal focal hypertrophy of the adrenal cortex (arrows) is present, which involves the zona glomerulosa with extension into the zona fasciculata. B) This minimal focus of hypertrophy (arrow) was confined to the zona fasciculata. C) A large area of hypertrophy (arrows) is evident; many of the hypertrophied cells also have pronounced vacuolation.

Testes: The incidence of interstitial cell hyperplasia showed a statistically significant positive trend, but there were no significant pairwise comparisons of the exposed groups to the control group (Table 14; Appendix H). Interstitial cell hyperplasia consisted of focal proliferations of interstitial cells that did not cause compression of surrounding seminiferous tubules. Minimal lesions were typically smaller than the size of one seminiferous tubule, whereas mild lesions were somewhat larger, but still did not cause compression. All lesions were unilateral and no interstitial cell adenomas were observed in this study.

The incidence of fibrinoid necrosis of the arterioles was significantly increased in 10,000 ppm males compared to the control group (Table 14; Appendix H). Arteriole fibrinoid necrosis was characterized by vessels with a tunica media thickened by amorphous eosinophilic material. Severity grading was based on the percentage of arterioles having fibrinoid necrosis of the vessel walls: Grade 1 (minimal) was used when up to 25% of the arterioles in the testes were affected; Grade 2 (mild) when 25–50% of the arterioles were affected; Grade 3 (moderate) when 50–75% of the arterioles were affected, and Grade 4 (marked) when greater than 75% of the arterioles

in the testes had fibrinoid necrosis of the vascular walls. Fibrinoid necrosis of these blood vessels was often accompanied by a mixed inflammatory cell infiltrate and was frequently found in vessels in other tissues as well. Polyarteritis nodosa involving necrosis and inflammation of arterioles and arteries is a common spontaneous age-related lesion in several strains of rats.[104]

Pancreas: The incidence of chronic active inflammation affecting the arterioles was significantly increased in 1,000 ppm males compared to the control group at the end of the 2-year study (Table 14; Appendix H). This lesion was not considered to be a primary pancreatic lesion, but rather, part of the syndrome of inflammation and necrosis of the arteries and arterioles (polyarteritis nodosa) that develops in certain strains of rats. Arteries and arterioles commonly involved in polyarteritis nodosa include those in the pancreas, mesentery, and testes.[105] In this particular case, although the incidences of chronic active inflammation of the arterioles were increased in the 1,000 and 10,000 ppm males, the incidence of chronic active inflammation of the pancreatic arteries was not statistically different from the control group in any exposed group. The arteries, larger than the arterioles, would be easier to observe in sections of tissue and because almost every male rat that had inflammation recorded in the arterioles of the pancreas also had inflammation of the arteries of the pancreas (the exception being three 1,000 ppm males) the differences in the incidences of arteriole inflammation might simply reflect fewer observed cross sections of the smaller arterioles in some rats.

Mammary gland: The incidences of mammary gland fibroadenoma and carcinoma were significantly decreased, relative to the control group, in 10,000 ppm females at the end of the 2-year study (fibroadenoma: 32/50, 30/50, 27/50, 18/50; carcinoma: 7/50, 5/50, 7/50, 1/50; Appendix H).

Mice

Two-year Study

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 15 and in the Kaplan-Meier survival curves (Figure 12). Survival of all exposed groups of male and female mice was not significantly different from that of the control groups.

Table 15. Survival of Mice in the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male				
Animals Initially in Study	50	50	50	50
Missing[a]	1	0	0	0
Moribund	2	1	2	0
Natural Deaths	13	9	5	8
Animals Surviving to Study Termination	34	40	43	42
Percent Probability of Survival at End of Study[b]	69	80	86	84
Mean Survival (Days)[c]	691	715	701	688
Survival Analysis[d]	p = 0.266N	p = 0.251N	p = 0.094N	p = 0.155N
Female				
Animals Initially in Study	50	50	50	50
Moribund	1	5	2	1
Natural Deaths	7	6	4	3
Animals Surviving to Study Termination	42	39[e]	44	46
Percent Probability of Survival at End of Study[b]	84	78	88	92
Mean Survival (Days)[c]	716	696	720	717
Survival Analysis[d]	p = 0.135N	p = 0.492	p = 0.724N	p = 0.369N

[a]Censored in the survival analysis.
[b]Kaplan-Meier determinations.
[c]Mean of all deaths (uncensored, censored, and study termination).
[d]The result of the life-table trend test[72] is in the control column, and the results of the life-table pairwise comparisons[73] with the control group are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by N.
[e]Includes one animal that died during the last week of the study.

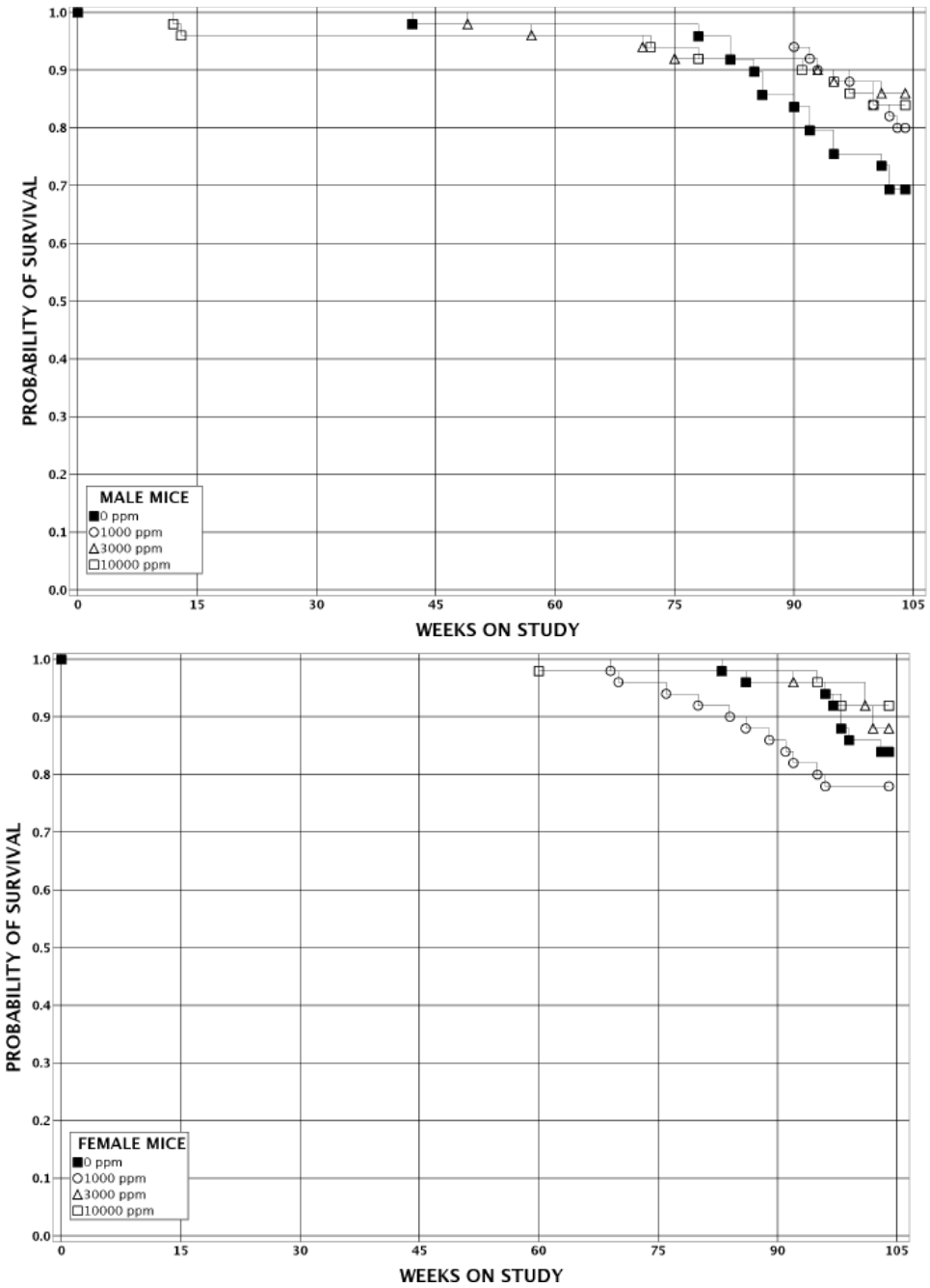


Figure 12. Kaplan-Meier Survival Curves for Mice Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed for Two Years

Body Weights, Feed and Compound Consumption, and Clinical Observations

Mean body weights of 1,000 and 3,000 ppm males and females were within 10% of those of the control groups throughout the study (Table 16, Table 17; Figure 13). Mean body weights of 10,000 ppm males and females were at least 10% less than those of the control groups generally at weeks 17 and 12, respectively. Feed consumption by exposed groups of males and females was not significantly different from that by the control groups (Appendix H). Dietary concentrations of 1,000, 3,000, and 10,000 ppm resulted in average daily doses of approximately 113, 339, and 1,207 mg 2H4MBP /kg body weight for males and 109, 320, and 1,278 mg/kg for females. No clinical findings in exposed groups of male or female mice were considered to be related to 2H4MBP exposure (Appendix H).

Table 16. Mean Body Weights and Survival of Male Mice in the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

Day	0 ppm		1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	21.8	50	21.8	100	50	21.9	100	50	21.9	100	50
8	22.6	50	22.3	99	50	22.4	99	50	21.9	97	50
15	23.0	50	22.9	100	50	23.0	100	50	22.4	97	50
22	23.4	50	23.4	100	50	23.3	100	50	22.5	96	50
29	24.2	50	24.4	101	50	23.9	99	50	23.1	95	50
36	25.0	50	25.4	102	50	24.8	99	50	23.7	95	50
43	25.5	50	26.0	102	50	25.3	99	50	24.2	95	50
50	26.5	50	26.7	101	50	25.8	97	50	24.9	94	50
57	27.1	50	27.3	101	50	26.3	97	50	25.3	93	50
64	27.9	50	28.3	101	50	26.9	97	50	25.9	93	50
71	28.4	50	28.8	101	50	27.8	98	50	26.7	94	50
78	29.1	50	29.4	101	50	28.3	97	50	26.6	92	49
85	28.9	50	29.3	101	50	27.9	97	50	26.5	92	49
92	30.0	50	30.2	101	50	28.4	95	50	27.2	91	48
120	33.3	50	33.4	100	50	31.6	95	50	29.4	88	48
148	35.3	50	35.0	99	50	33.2	94	50	30.0	85	48
176	37.5	50	37.6	100	50	35.0	93	50	31.4	84	48
204	40.4	50	40.6	100	50	37.8	94	50	33.5	83	48
232	42.5	50	42.2	99	50	39.8	94	50	35.1	83	48
260	43.6	50	43.2	99	50	41.0	94	50	35.9	83	48
288	43.6	49	42.9	98	50	42.0	96	50	36.8	84	48
316	45.5	49	44.4	98	50	43.1	95	50	37.6	83	48
344	46.3	49	45.9	99	50	44.1	95	49	38.6	83	48
372	45.6	49	44.9	99	50	43.4	95	49	38.1	84	48
400	46.7	49	46.6	100	50	44.7	96	48	39.3	84	48
428	46.0	49	46.2	100	50	44.6	97	48	39.0	85	48
456	46.4	49	46.7	101	50	44.7	96	48	38.9	84	48
484	45.9	49	46.2	101	50	44.7	97	48	38.9	85	48
512	46.9	49	46.5	99	50	45.5	97	47	39.1	83	47
540	47.4	49	47.4	100	50	46.6	98	46	40.7	86	47
568	47.2	48	46.8	99	50	45.9	97	46	40.1	85	46
596	47.6	45	47.1	99	50	46.6	98	46	40.6	85	46
624	47.2	42	45.7	97	48	45.0	96	46	39.2	83	46
652	46.6	39	46.1	99	45	45.0	97	45	39.1	84	45
680	46.8	37	43.9	94	44	45.3	97	44	38.7	83	43
708	45.8	36	44.6	97	42	45.5	99	43	38.8	85	42
Mean for Weeks											
1–13	25.6	–	25.8	101	–	25.2	98	–	24.3	95	–
14–52	39.8	–	39.5	99	–	37.6	94	–	33.6	85	–
53–102	46.6	–	46.1	99	–	45.2	97	–	39.3	84	–

Table 17. Mean Body Weights and Survival of Female Mice in the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

Day	0 ppm		1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	18.0	50	17.7	98	50	17.9	99	50	17.7	99	50
8	19.0	50	18.7	98	50	18.8	99	50	18.1	96	50
15	20.0	50	19.6	98	50	19.6	98	50	19.0	95	50
22	20.8	50	20.6	99	50	20.3	98	50	19.4	94	50
29	21.7	50	21.3	98	50	20.8	96	50	20.2	93	50
36	22.5	50	22.1	98	50	21.9	97	50	20.9	93	50
43	22.9	50	22.5	98	50	22.6	99	50	21.2	93	50
50	23.6	50	22.9	97	50	23.0	98	50	21.8	93	50
57	24.5	50	23.9	98	50	23.9	97	50	22.6	92	50
64	24.8	50	24.3	98	50	24.7	100	50	22.8	92	50
71	25.7	50	25.3	98	50	24.9	97	50	23.1	90	50
78	25.5	50	25.4	100	50	25.3	99	50	23.3	91	50
85	26.5	50	25.9	98	50	26.1	98	50	23.8	90	50
92	27.1	50	26.0	96	50	26.3	97	50	23.8	88	50
120	29.8	50	29.2	98	50	28.6	96	50	25.1	84	50
148	31.9	50	30.7	96	50	31.1	98	50	26.3	82	50
176	34.0	50	33.3	98	50	32.2	95	50	26.9	79	50
204	36.8	50	35.1	96	50	36.0	98	50	27.6	75	50
232	38.6	50	37.0	96	50	37.1	96	50	29.7	77	50
260	39.9	50	38.9	97	50	39.6	99	50	30.5	76	50
288	42.3	50	40.3	95	50	40.8	96	50	31.5	75	50
316	44.3	50	42.6	96	50	42.4	96	50	33.2	75	50
344	45.2	50	44.3	98	50	43.9	97	50	34.2	76	50
372	46.0	50	44.3	96	50	45.1	98	50	34.7	75	50
400	47.7	50	45.8	96	50	46.9	98	50	36.1	76	50
428	48.7	50	47.2	97	50	46.5	95	50	36.7	75	49
456	48.9	50	47.9	98	50	46.6	95	50	36.7	75	49
484	52.0	50	49.4	95	48	49.5	95	50	37.7	72	49
512	53.2	50	49.2	93	48	50.5	95	50	38.4	72	49
540	54.5	50	51.4	94	47	51.8	95	50	39.3	72	49
568	54.3	50	51.7	95	46	50.9	94	50	39.3	72	49
596	54.7	48	51.6	94	45	49.5	91	49	39.2	72	49
624	54.2	48	51.2	94	43	50.3	93	49	39.3	72	49
652	52.3	48	50.2	96	41	49.8	95	48	38.9	74	49
680	52.5	46	51.0	97	39	50.1	96	48	39.5	75	47
708	52.5	43	50.3	96	39	47.2	90	46	39.2	75	46
Mean for Weeks											
1–13	22.7	–	22.3	98	–	22.3	98	–	21.1	93	–
14–52	37.0	–	35.7	97	–	35.8	97	–	28.9	79	–
53–102	51.7	–	49.3	96	–	48.8	95	–	38.1	74	–

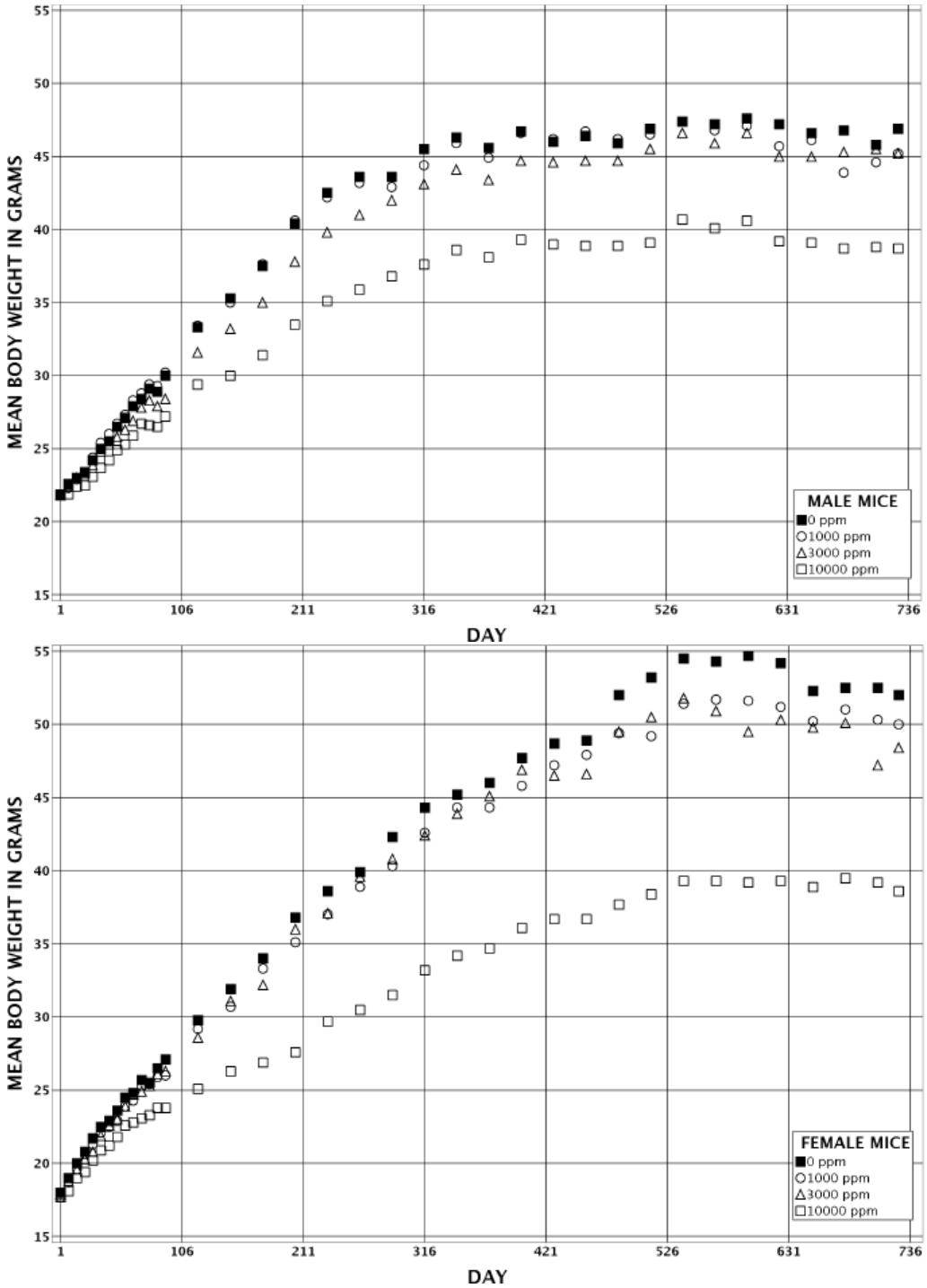


Figure 13. Growth Curves for Mice Exposed to 2-Hydroxy-4-methoxybenzophenone in Feed for Two Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidence of neoplasms and nonneoplastic lesions of the hematopoietic tissues (bone marrow and spleen), liver, kidney, Harderian gland, pituitary gland (pars distalis), thyroid gland, pancreatic islets, and adrenal cortex. Summaries of the incidence of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms are presented in Appendix H.

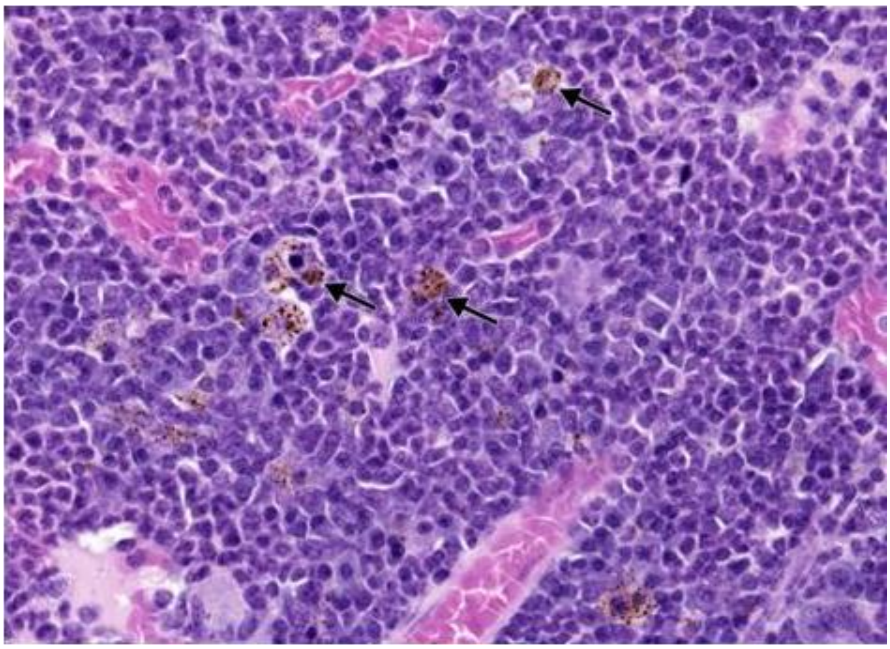
Hematopoietic tissues (bone marrow and spleen): The incidences of pigment in the bone marrow were significantly increased in 10,000 ppm males and females when compared to their respective control group (Table 18; Appendix H). Also relative to their respective control group, the incidences of pigment in the spleen were significantly increased in 10,000 ppm males and 3,000 and 10,000 ppm females (Table 18; Appendix H). In both the bone marrow and spleen, the pigment was characterized by a golden-brown to brown granular material found within the cytoplasm of macrophages (Figure 14 and Figure 15). It was consistent with hemosiderin, and the presence of iron was confirmed using a Perl's stain on spleens from several animals (Figure 16). Some amount of pigment could be found in most animals, but pigment was only recorded as a change when it was above the threshold of that which was considered a normal background level. Typically, pigment was recorded when there were increased numbers of pigment-containing macrophages, often present in clusters of up to 10 or more macrophages, and the macrophages individually contained more pigment than what was observed at background levels. All findings of pigment in the bone marrow were of minimal severity. The majority of lesions in the spleen were of minimal severity, with only one animal each having lesions of mild or moderate severity. Mild and moderate pigment was based on a subjective increase in the amount of pigment compared to observations of minimal severity.

Table 18. Incidences of Nonneoplastic Lesions of the Bone Marrow, Spleen, Liver, and Kidney of Mice in the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male				
Bone Marrow[a]	47	48	48	50
Pigment[b]	3** (1.0)[c]	2 (1.0)	9 (1.0)	50** (1.0)
Spleen	48	50	49	50
Pigment	4** (1.0)	5 (1.0)	10 (1.0)	17** (1.0)
Liver	49	50	50	50
Hepatocyte, syncytial alteration	2** (1.0)	39** (1.0)	45** (1.5)	48** (1.8)
Kidney	48	50	50	50
Renal tubule, cytoplasmic alteration	0**	0	0	46** (2.0)
Infiltration cellular, lymphocyte	40* (1.0)	40 (1.0)	43 (1.0)	46* (1.0)
Nephropathy, chronic progressive	41* (1.1)	48 (1.1)	48* (1.0)	50* (1.1)
Female				
Bone Marrow	49	50	50	50
Pigment	6** (1.0)	0*	0*	50** (1.0)
Spleen	49	50	49	50
Pigment	12** (1.0)	10 (1.0)	36** (1.0)	38** (1.0)
Kidney	49	50	50	50
Metaplasia, osseous	0*	1	3	5*

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$ by the Poly-3 test; **$p \leq 0.01$.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked).

A)



B)

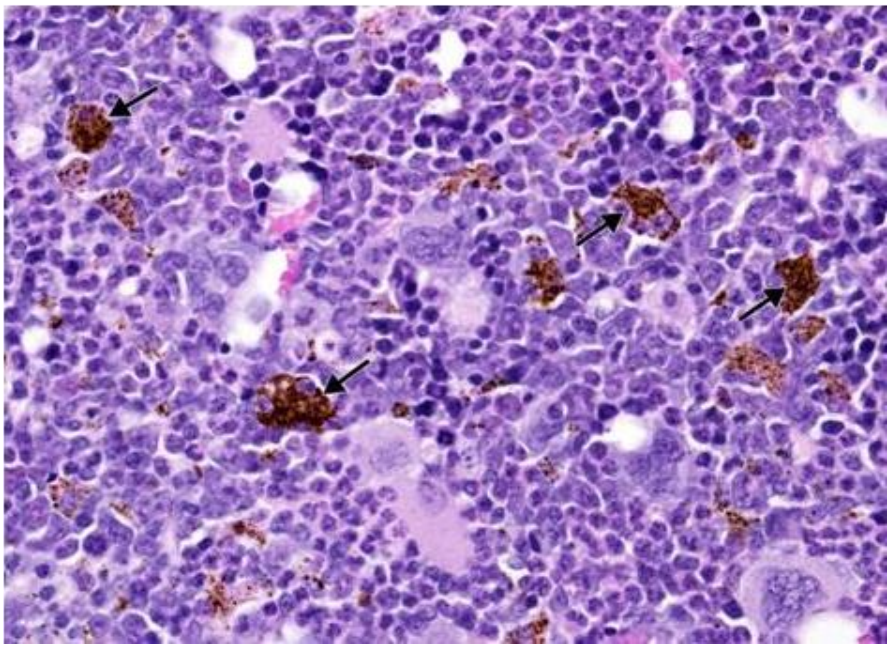
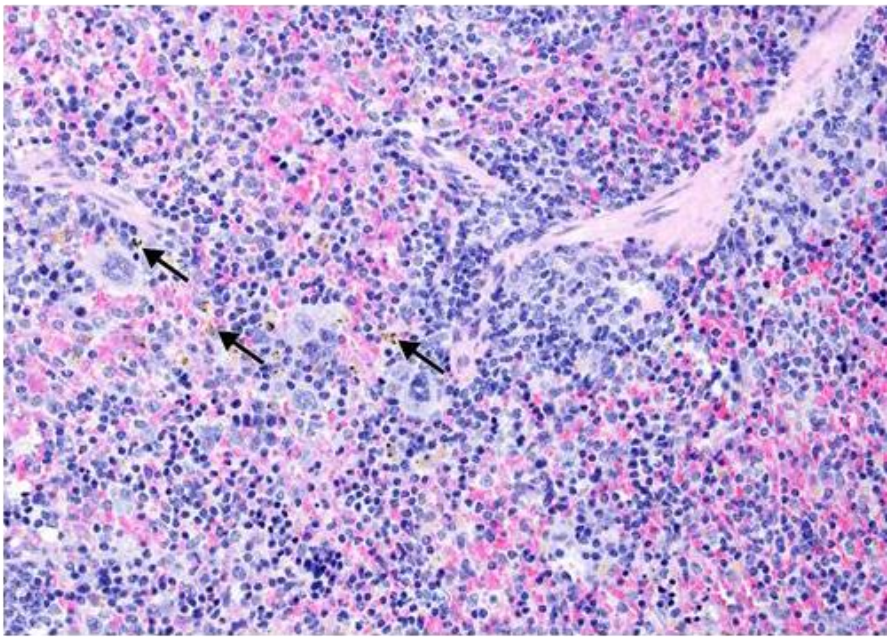


Figure 14. Pigment in the Bone Marrow from Female B6C3F1/N Mice Exposed to 0 or 10,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) A background level of pigment appears within macrophages in the bone marrow (arrows) of a control mouse. B) In a female mouse exposed to 10,000 ppm 2-hydroxy-4-methoxybenzophenone, an increased amount of pigment in the macrophages in the bone marrow (arrows) is apparent.

A)



B)

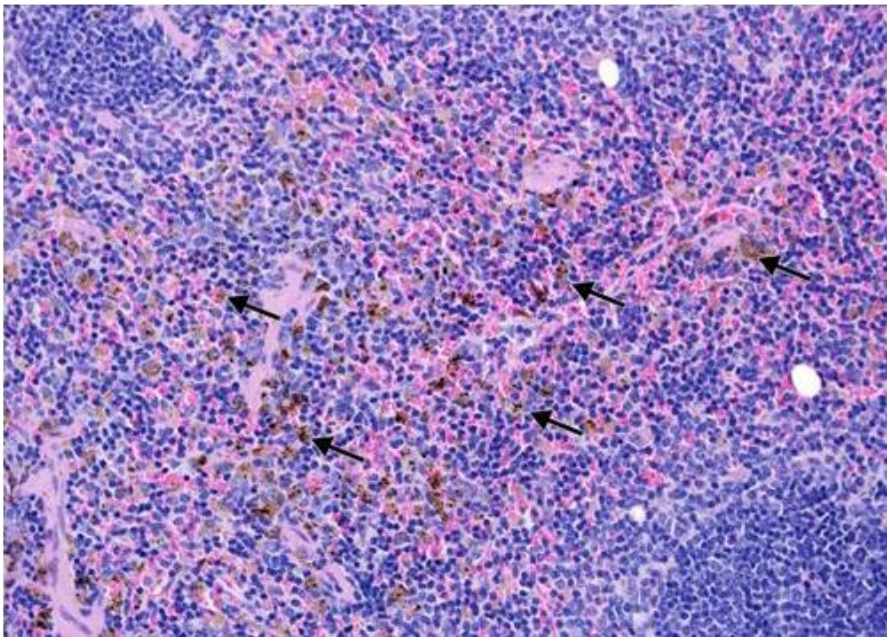
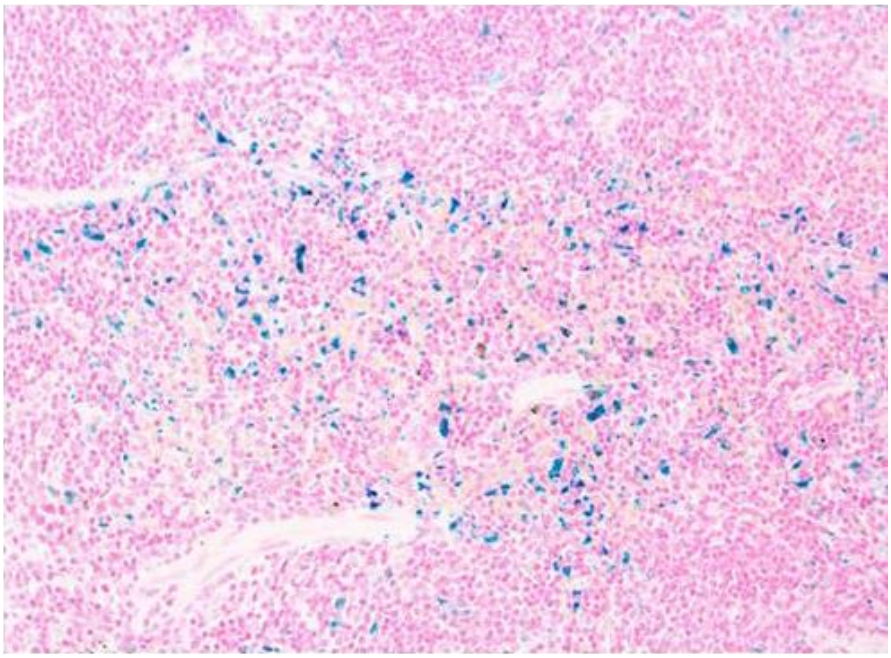


Figure 15. Pigment in the Spleen from Female B6C3F1/N Mice Exposed to 0 or 10,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

A) Similar to what was seen in the bone marrow, some pigment is present in control animals as a background observation (arrows). B) An increased amount of pigment is evident in the spleen (arrows) in a female mouse exposed to 10,000 ppm 2-hydroxy-4-methoxybenzophenone when compared to the spleen from the control mouse.

A)



B)

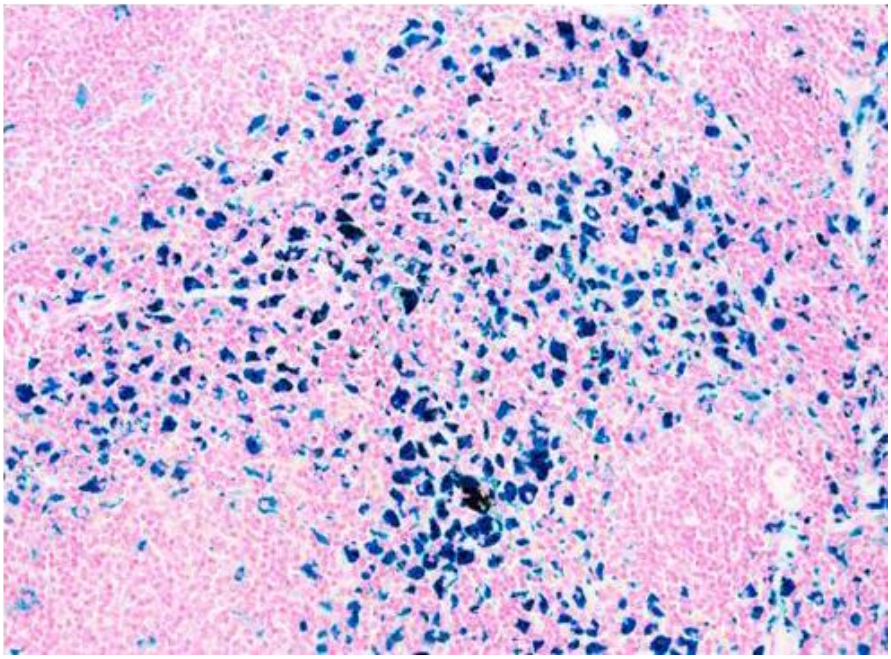


Figure 16. Pigment in the Spleen from Female B6C3F1/N Mice Exposed to 0 or 10,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (Perl's Iron Stain)

A) The positive (blue) staining indicates that the pigment in the spleen contains iron, which is consistent with hemosiderin from a control group mouse. B) Increased iron-containing pigment in spleen of female mouse exposed to 10,000 ppm 2-hydroxy-4-methoxybenzophenone; compare the amount of positive (blue) staining in this photomicrograph to that in panel A.

Liver: The incidences of hepatocyte syncytial alteration were significantly increased in all exposed groups of males compared to the control group (Table 18; Appendix H).

Hepatocytes with two or three nuclei are common in old mice and were not included in the diagnosis of syncytial alteration. Hepatocytes recorded with syncytial alteration had four to seven or more nuclei. Affected hepatocytes were larger than normal, but the individual nuclei were typically small and densely basophilic (Figure 17). Severity grading was based upon the number of altered hepatocytes observed within a section of liver, with minimal (1) being recorded when one to three such hepatocytes were observed; mild (2) severity being recorded when four to 10 syncytial cells were observed; and moderate (3) severity being recorded when >10 were observed. No marked lesions were recorded.

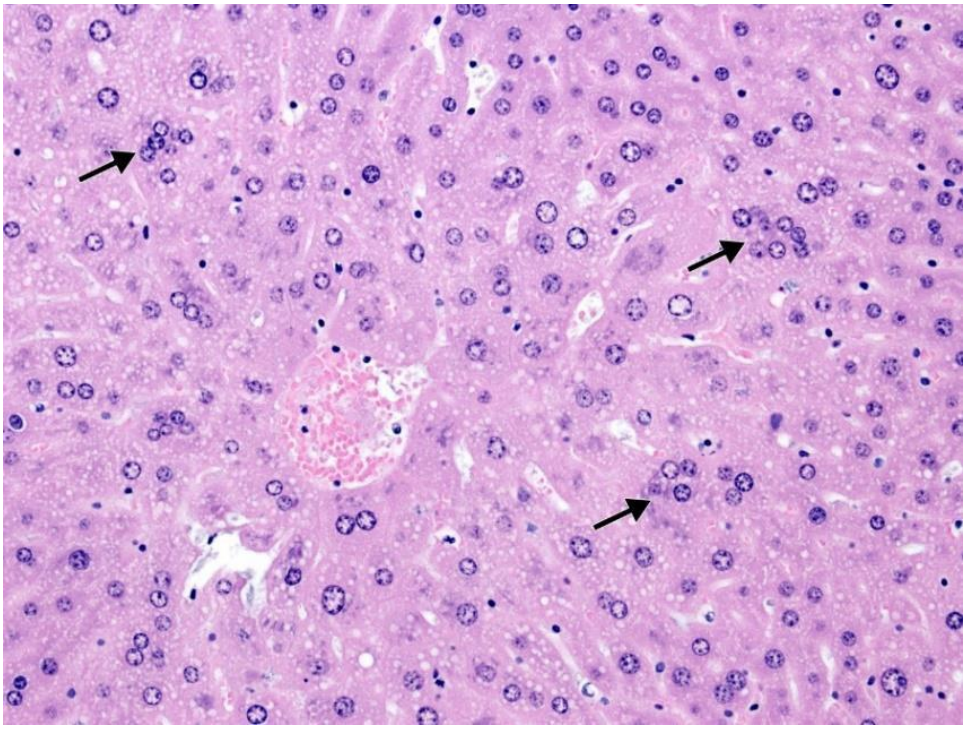


Figure 17. Syncytial Alteration in the Liver from a Male B6C3F1/N Mouse Exposed to 10,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

This animal died on day 545 of the 2-year study. Enlarged hepatocytes contain multiple nuclei (arrows).

Kidney: The incidence of renal tubule cytoplasmic alteration was significantly increased in the 10,000 ppm males compared to the control group (Table 18; Appendix H). Vacuolation of the renal tubules, typically in the outer cortex, is a normal background finding in male mice (Figure 18). The presence of these vacuoles is not recorded as a lesion; however, when a decrease in the vacuolation of the renal tubules is noted, it is typically recorded. In the current study, the normal background vacuolation decreased in renal tubules in the 10,000 ppm males, and this was recorded as an increased incidence of cytoplasmic alteration. (Figure 18).

Incidences of chronic progressive nephropathy were significantly increased in 3,000 and 10,000 ppm males compared to the control group In addition, the incidence of lymphocytic cellular infiltration were significantly increased in 10,000 ppm males compared to the control

group (Table 18; Appendix H). Lymphocytic cellular infiltration was characterized by increased numbers of lymphocytes within the interstitium of the kidney.

Lymphocytic infiltrates are very common in aged mouse kidneys, and lymphocytes can also be associated with chronic progressive nephropathy. Therefore, lymphocytic cellular infiltration was not diagnosed unless there were larger accumulations of lymphocytes than is typically seen as a background change or what one would expect associated with chronic progressive nephropathy. Early and minimal chronic progressive nephropathy was characterized by basophilic, hyperplastic tubules with thickened basement membranes (Figure 18). Later and more severe changes were interstitial infiltrations of mononuclear cells (predominantly lymphocytes), hyaline casts, dilated tubules, and interstitial fibrosis. The vast majority of the occurrences of chronic progressive nephropathy were of minimal severity, with only a few mild or moderate (and one marked occurrence) scattered among the exposure groups.

The incidence of osseous metaplasia was significantly increased in 10,000 ppm females compared to the control group (Table 18; Appendix H). This lesion consisted of small areas of mature bone within the cortex of the kidney. Osseous metaplasia is regarded as a spontaneous lesion with unknown pathological significance.

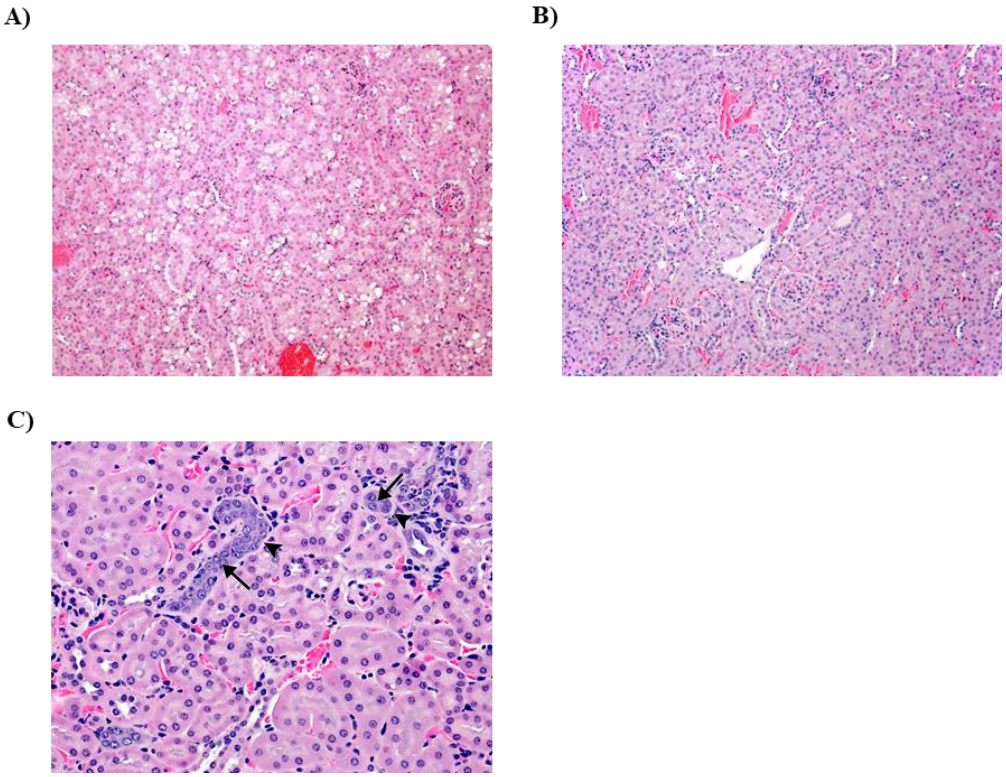


Figure 18. Renal Tubule Vacuolation in the Kidney from Male B6C3F1/N Mice Exposed to 0 or 10,000 ppm 2-Hydroxy-4-methoxybenzophenone for Two Years (H&E)

Vacuolation of the renal tubules, typically in the outer cortex, is a normal background finding in control male mice. B) The renal tubules of a male mouse exposed to 10,000 ppm 2-hydroxy-4-methoxybenzophenone show a lack of vacuoles (cytoplasmic vacuolation) compared to what is typically seen in male mice. C) Basophilic tubules (arrows) with thickened basement membranes (arrowheads) are evidence of minimal chronic progressive nephropathy in a male mouse exposed to 10,000 ppm 2-hydroxy-4-methoxybenzophenone. In addition, the normal vacuoles seen in the renal tubules of male mice are not present (cytoplasmic alteration).

Other tissues: In the Harderian gland, the incidence of adenoma was significantly increased in 1,000 ppm females compared to the control group (1/50, 6/50, 4/50, 3/50; Appendix H). None of these values was outside the historical control range (33/640; range: 2% to 18%),.

Relative to the control group, significantly increased incidences of focal hyperplasia in the pituitary gland (pars distalis) (15/47, 24/49, 14/47, 17/50) and follicular degeneration in the thyroid gland (20/48, 30/49, 18/48, 20/48) occurred in 1,000 ppm females (Appendix H).

In the pancreatic islets, significantly decreased incidences of hyperplasia occurred in 10,000 ppm males (29/47, 31/50, 25/48, 20/50) and 1,000 and 3,000 ppm females compared to their respective control group (11/49, 3/49, 4/49, 7/49) (Appendix H).

In the adrenal cortex, the incidences of accessory adrenal cortical nodules in 1,000 and 3,000 ppm females were significantly less than that in the control group (9/50, 2/50, 2/50, 4/50; Appendix H).

None of these lesions was considered toxicologically significant.

Genetic Toxicology

2H4MBP (20 to 6,000 μg/plate) was not mutagenic in *Salmonella typhimurium* strains TA98 or TA100 or *Escherichia coli* strain WP2 *uvrA*/pKM101 when tested with and without 10% rat liver metabolic activation enzymes (S9) (Table A-1).

Discussion

These National Toxicology Program (NTP) dietary studies evaluated the toxic and carcinogenic potential of 2H4MBP. Two-year studies in Hsd:Sprague Dawley® SD® rats, with exposure beginning in utero, and in B6C3F1/N mice were conducted at exposure concentrations of 0, 1,000, 3,000, and 10,000 ppm 2H4MBP in feed. The initiation of F_1 generation exposure on GD 6 (after expected implantation) in the 2-year rat study was selected to reflect potential human exposure to 2H4MBP, often present in sunscreens and cosmetics, which can occur at all human life stages, including in utero and early life.[106] Information from previous NTP studies[107; 32] was used to aid in selection of exposure concentrations for the 2-year study. An interim evaluation at 14 weeks on a subset of rats in the control and 10,000 ppm exposure groups was conducted to confirm expected responses. The oral route of administration was selected on the basis of NTP studies that used radiolabeled 2H4MBP and that demonstrated disposition is similar between oral and dermal administration.[15; 22] Moreover, given the pragmatic challenges of topical application in young small rodents, which requires the shaving of fur, and inter- and intra-animal grooming, exposure via dietary feed was selected.

Postweaning dietary 2H4MBP was well tolerated in rats and did not adversely affect survival. Consumption of 2H4MBP in the 1,000, 3,000, and 10,000 ppm groups resulted in average daily 2H4MBP doses of approximately 58, 168, and 585 mg 2H4MBP /kg body weight for males and 60, 180, and 632 mg/kg for females. These doses translate to a range of 348 to 3,792 mg/m^2 , using a K_m (body weight [kg]/surface area [m^2]) value of 6 for rats[108] and are only 5- to 57-fold higher than those used in a repeated dermal application study (20 g/m^2) study in postmenopausal women.[109] NTP studies have shown previously that using plasma concentrations of 2H4MBP in rats following feed exposure of 3,000–30,000 ppm compared to humans following repeated dermal application of 20 g/m^2 rat: human dose multiples were 0.1 to 4.[22] Human geometric mean maximum plasma concentrations of 2H4MBP have been shown to be approximately 200 ng/mL when topically applied. This exceeds the FDA guidance of 0.5 ng/mL that would necessitate the conduct of additional nonclinical toxicity studies.[110] Collectively, these data demonstrate similar external (5- to 57-fold) and internal (0.1- to 4-fold) exposure of 2H4MBP in rats and humans. However, the effects on body weight observed in both rats and mice precluded any higher dose levels in a chronic study.

The 14-week interim evaluation was conducted to ensure that there were no unexpected strain differences in response to 2H4MBP in feed; this interim evaluation observed similar magnitude of 2H4MBP-induced effects on female body weight in Sprague Dawley rats exposed to 10,000 ppm 2H4MBP compared to that observed in female F344/N rats exposed to 12,500 ppm 2H4MBP in the diet (12% versus 11%, respectively).[32] F334/N male rat body weights were not affected at 12,500 ppm 2H4MBP, consistent with what was observed in Sprague Dawley males in the current study (Appendix H).[32] The observed increases in absolute and relative kidney weights in male Sprague Dawley rats and decrease in absolute kidney weight in female Sprague Dawley rats exposed to 10,000 ppm 2H4MBP were not observed in the F344/N rats exposed to 12,500 ppm 2H4MBP (Appendix H).[32] However, increases in absolute and relative kidney weights were observed in male F344/N rats exposed to 50,000 ppm 2H4MBP, and females exposed to 25,000 or 50,000 ppm 2H4MBP in feed.[32] The absence of histopathological findings in the kidneys of Sprague Dawley rats at the interim evaluation is also consistent with what was

previously observed at 12,500 ppm in F344/N male and female rats (Appendix H).[32] The observed increase (~20%) in absolute and relative male Sprague Dawley rat liver weights is fairly consistent with what was previously observed in male F344/N rats (26% and 31%, respectively) exposed to 12,500 ppm (Appendix H).[32] The increase in relative liver weight was comparable to that observed in the female F334/N rats exposed to 12,500 ppm (Appendix H).[32] In general, 2H4MBP-induced responses in the Sprague Dawley rats were consistent with those observed previously in the F344/N rats.

At the 14-week interim evaluation in rats, the observed increase in liver weight in males exposed to 10,000 ppm 2H4MBP occurred concomitantly with a significantly increased incidence of mixed-cell cellular infiltration. However, it is unlikely that the cellular infiltrates, which were all of minimal severity, would be responsible for the changes in the liver weights observed in male rats at this time point. 2H4MBP exposure has been shown to increase hepatic *Cyp1a1* and *Cyp4a* expression the magnitude of which is consistent with enzyme induction and moderate increase in tissue weight. In the NTP Modified One-Generation (MOG)[111] study, focal inflammation was observed in all F_1 male exposure groups in the reproductive performance cohort (~8–25%), including the control group (7%), with the low exposure group of 3,000 ppm displaying the highest incidence (10/40). The incidence in F_1 females was 5% at 30,000 ppm, the only 2H4MBP exposure group examined.

The absence of appreciable alterations in the kidneys after 14 weeks of 2H4MBP exposure was somewhat unexpected given the previous reports of 2H4MBP-induced kidney histopathology in male F344/N rats at exposure concentrations ≥12,500 ppm in the diet.[32] This could be a function of dose, given that in the NTP MOG study, a dietary concentration of 10,000 ppm—the highest concentration used in the current study—did not result in apparent 2H4MBP-related adverse histopathological kidney findings following a similar duration of exposure. However, rats exposed to 30,000 ppm in the MOG study did display 2H4MBP-related kidney findings in renal tubules and pelvises (concretions, epithelial degeneration, and dilatation).

In the 2-year study, the incidences of malignant meningiomas in the brain in male rats were higher in the 1,000 and 3,000 ppm groups (1/50 [1/25 litters]; 3/50 [2/25 litters]) than in the control group (0/50 [0/29 litters]) or in the 10,000 ppm group (0/50 [0/28 litters]). In addition, one malignant meningioma was diagnosed in the spinal cord of a male rat in the 3,000 ppm group. Spinal cords were only examined from animals displaying clinical neurologic signs, however, so it is possible that the true incidence of spinal cord neoplasms was underreported. Although not dose responsive, these incidences of malignant meningiomas exceeded previous historical control incidences (none was observed in the control groups from six previously conducted studies) and therefore was considered equivocal evidence of carcinogenic activity. This conclusion is consistent with conclusions summarized in a previous NTP technical report (TR 356 – Toxicology and Carcinogenesis Studies of Furosemide) that found three meningiomas, all in male rats from the low-dose group, and this was considered equivocal evidence of carcinogenic activity.[112]

Although the malignant meningiomas observed in the brain in this study lacked features resembling granular cells tumors sometimes seen in benign meningiomas, the possibility that some meningiomas and granular cell tumors share a common progenitor is noteworthy. However, whereas benign granular cell tumors have been recorded in the historical controls for this study, malignant meningiomas have not. Therefore, although it may be beneficial to consider

the combination of granular cell tumors and meningiomas, it is also necessary to consider malignant meningiomas on their own for the interpretation of the data in this study.

The incidences of thyroid gland C-cell adenoma in all groups of female rats exposed to dietary 2H4MBP for 2 years were higher than that in the control group, but the occurrences were not related to exposure concentration, and the higher incidence was only statistically significant at 3,000 ppm: 5/50 (5/29 litters) in the control group; 11/50 (10/25 litters); 17/50 (15/25 litters); and 10/50 (9/29 litters) in the 1,000, 3,000, and 10,000 ppm groups, respectively. This incidence exceeded the historical control incidence of recent NTP studies (38/339; range: 4% to 22%).

Along with the lack of a dose response, there were no increases in the incidences of thyroid gland C-cell hyperplasia or C-cell carcinomas when compared to the control group, nor was there any support from the males concerning proliferative lesions of the C-cells. Therefore, it was considered that thyroid gland C-cell adenomas in female rats may have been related to 2H4MBP exposure.

Female rats exposed to all dietary concentrations of 2H4MBP for 2 years had increased incidences of uterine stromal polyps (15/50 [12/25 litters]; 18/50 [16/25 litters]; 10/50 [10/29 litters]) relative to the control group (8/50 [8/29 litters]); the 3,000 ppm group was the only group that displayed a statistically significant difference from the control group. While stromal sarcomas only occurred in 2H4MBP exposed groups and not in the control group, the numbers of stromal sarcomas were not significantly different between the control group and any of the exposed groups; hence, it was considered that the increased incidences of uterine stromal polyps may have been related to 2H4MBP exposure.

The 3,000 ppm females also had a significantly higher incidence of atypical endometrial hyperplasia relative to the control group, which is considered a preneoplastic lesion of the uterine epithelium. However, that exposure group had a significantly lower incidence of uterine adenocarcinomas. These findings are inconsistent with the purported 2H4MBP estrogenic activity given that ultra-low doses (2–50 parts per billion [ppb]) of ethinyl estradiol have shown to be associated with increased incidences of both atypical uterine focal hyperplasia (6/51, 14/50, 16/49, 20/50) and squamous metaplasia (2/51, 6/50, 8/49, 13/50) in female Sprague Dawley rats.[113]

In the 2-year rat study, the incidence of mammary gland fibroadenoma in the female 10,000 ppm group (18/50 [18/29 litters]) was significantly lower than the control incidence (32/50 [23/29 litters]). Similarly, the 10,000 ppm group displayed a lower incidence of mammary gland carcinoma (1/50 [1/29 litters]) than did the control group (7/50 [6/29 litters]). The concurrent control incidence of fibroadenomas is higher than recent historical control incidences (195/340; range: 40% to 70%). The incidence of mammary gland carcinomas in the concurrent control is also higher than recent historical control incidences (30/340; range: 4% to 14%). The 10,000 ppm females had a mean body weight at terminal sacrifice that was 76% of the control group, and this lower body weight might have contributed to the lower incidences of mammary gland carcinomas and fibroadenomas in this group.[114] These 2H4MBP findings are also inconsistent with the purported 2H4MBP estrogenic activity. Exposure to ultra-low dose (2–50 ppb) ethinyl estradiol has been reported to be associated with an increased incidence of mammary alveolar or ductal hyperplasia in male Sprague Dawley rats (1/44, 4/45, 8/47,

21/44).[113] No changes in the incidences of mammary gland lesions were observed in males exposed to 2H4MBP.

At the end of the 2-year study, the incidences of focal hypertrophy within the adrenal cortex were significantly higher in females exposed to 1,000 (42/50) or 3,000 ppm (39/50) 2H4MBP relative to the control group (24/50). These findings were not associated with an increased incidence of adrenocortical neoplasia.

In male rats, there was an increase in the incidence of interstitial cell hyperplasia of the testes in the 10,000 ppm group, but the increase was not statistically different from controls with a pairwise comparison. No interstitial cell adenomas were observed in male rats of any group in the study.

The 2-year study was conducted in B6C3F1/N mice at dietary exposure concentrations of 0, 1,000, 3,000, and 10,000 ppm, which resulted in average daily doses of approximately 113, 339, and 1,207 mg 2H4MBP/kg body weight for males and 109, 320, and 1,278 mg/kg for females. 2H4MBP exposure did not affect survival of either sex. After several months of 2H4MBP exposure, both males and females in the 10,000 ppm groups displayed lower body weights (>10%). No 2H4MBP-related increases in the incidences of neoplastic lesions were observed in the 2-year study.

Increased incidences of bone marrow pigment (hemosiderin) were observed in both male and female mice exposed to 10,000 ppm 2H4MBP for 2 years. The incidences of pigment in the spleen were higher in males and females exposed to 3,000 and 10,000 ppm 2H4MBP. Increased levels of hemosiderin in the spleen of mice can be associated with increased erythrocyte turnover.[115] In the current study there was no other evidence of an increase in the turnover of erythrocytes, such as changes in the cellularity of the bone marrow, or increased extramedullary hematopoiesis in the spleen. Additionally, there were no changes in hematology parameters or bone marrow histology in a 13-week dosed feed 2H4MBP mouse study.[32]

The incidences of hepatocyte syncytial alteration were significantly increased in all 2H4MBP-exposed groups of male mice. Ethylbenzene induced a similar response in the male mouse,[32] and these compounds share some structural similarities.[32] As with 2H4MBP no association was found between the syncytial cells and hepatocellular neoplasia in male mice in the ethylbenzene study, although there were increased incidences of hepatocellular tumors in female mice.

2H4MBP dietary exposure in male mice was associated with an increased incidence of renal tubule cytoplasmic alteration at 10,000 ppm. Vacuolation of the renal tubules, typically in the outer cortex, is a normal background finding in male mice.[116] The presence of these vacuoles is not recorded as a lesion; however, when a decrease in the vacuolation of the renal tubules is noted, it is typically recorded. In the current study, the normal background vacuolation decreased in renal tubules in the 10,000 ppm males, and this was recorded as an increased incidence of cytoplasmic alteration.

Conclusions

Under the conditions of these 2-year studies, there was *equivocal evidence of carcinogenic activity*[c] of 2H4MBP exposure in male Hsd:Sprague Dawley® SD® rats based on the occurrence of malignant meningiomas in the brain. There was *equivocal evidence of carcinogenic activity* in female Hsd:Sprague Dawley® SD® rats based on the increased incidence of thyroid C-cell adenomas and the increased incidence of uterine stromal polyps. There was *no evidence of carcinogenic activity* in male or female B6C3F1/N mice at exposure concentrations of 1,000, 3,000, and 10,000 ppm.

Increases in the incidences of nonneoplastic lesions of the testis in male rats and of the uterus and adrenal cortex in female rats occurred with exposure to 2H4MBP. Increases in the incidences of nonneoplastic lesions of the bone marrow (males and females), spleen (males and females), kidney (males and females), and liver (males) in mice occurred with exposure to 2H4MBP.

[c]See Explanation of Levels of Evidence of Carcinogenic Activity.

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Appendix A. Genetic Toxicology

Table of Contents

A.1. Bacterial Mutagenicity Test Protocol A-2
A.2. Results A-2

Tables

Table A-1. Mutagenicity of 2-Hydroxy-4-methoxybenzophenone in Bacterial Tester Strains A-3

A.1. Bacterial Mutagenicity Test Protocol

Testing procedures were modified from those originally reported by Zeiger et al.[64] Coded samples of 2-hydroxy-4-methoxybenzophenone (2H4MBP) (the same chemical lot that was used in the 2-year bioassays) were incubated with the *Salmonella typhimurium* (TA98, TA100) or *Escherichia coli* (WP2 *uvr*A pKM101) tester strains either in buffer or S9 mix (metabolic activation enzymes and cofactors from phenobarbital/benzoflavone-induced male Sprague Dawley rat liver) for 20 minutes at 37°C. Top agar supplemented with L-histidine (or tryptophan for the *E. coli* strain) and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- or tryptophan-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of 2H4MBP. The highest concentration tested was limited by toxicity in strain TA100; the other two strains were tested up to the assay limit dose of 6,000 µg/plate. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not related to dose, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. No minimum percentage or fold increase is required for a chemical to be judged positive or weakly positive, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

A.2. Results

2H4MBP (20 to 6,000 µg/plate) was not mutagenic in *S. typhimurium* strains TA98 or TA100 or *E. coli* strain WP2 *uvrA* pKM101 when tested with and without 10% rat liver metabolic activation enzymes (S9) (Table A-1).

Table A-1. Mutagenicity of 2-Hydroxy-4-methoxybenzophenone in Bacterial Tester Strains[a]

Strain	Concentration (µg/plate)	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA100					
	0	92.7 ± 4.1	111.7 ± 10.7	101.7 ± 1.3	156.3 ± 3.3
	20	95.0 ± 9.2	120.3 ± 9.8	–	–
	50	86.7 ± 2.9	115.0 ± 6.0	99.7 ± 1.9	157.7 ± 7.9
	125	82.3 ± 4.9	126.0 ± 4.6	88.0 ± 9.6	147.3 ± 10.3
	250	80.7 ± 4.3	83.3 ± 1.9[c]	76.7 ± 2.0	128.0 ± 15.2
	500	56.0 ± 4.0	51.3 ± 6.0[c]	58.3 ± 1.3	84.7 ± 6.7[c]
	1,000	14.3 ± 1.9[c]	54.3 ± 16.3[c]	25.7 ± 3.3[c]	55.7 ± 3.3[c]
	3,000	–	–	30.3 ± 3.0[c]	56.3 ± 3.4[c]
Trial Summary		Negative	Negative	Negative	Negative
Positive Control[b]		658.3 ± 14.4	573.3 ± 10.4	542.0 ± 9.5	554.0 ± 20.1
TA98					
	0	16.7 ± 2.9	17.7 ± 3.3	23.7 ± 4.6	22.0 ± 1.0
	125	9.0 ± 1.7	10.3 ± 3.7	14.7 ± 2.4	27.0 ± 2.3
	250	9.7 ± 0.7	15.7 ± 1.9	14.7 ± 0.7	17.7 ± 0.7
	500	6.0 ± 1.5	10.3 ± 1.9	11.3 ± 2.3	14.3 ± 0.9
	1,000	2.7 ± 0.3[c]	3.3 ± 0.3[c]	12.0 ± 2.3	13.0 ± 1.7[c]
	3,000	2.0 ± 0.6[c]	2.3 ± 1.3[c]	4.7 ± 1.5[c]	12.3 ± 1.8[c]
	6,000	2.3 ± 0.3[c]	9.7 ± 1.5[c]	10.7 ± 4.4[c]	17.3 ± 0.3[c]
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		537.7 ± 9.8	519.0 ± 24.6	1,485.7 ± 67.2	2,016.7 ± 34.7
Escherichia coli WP2 uvrA/pKM101					
	0	116.0 ± 11.5	109.3 ± 9.5	155.0 ± 9.3	152.7 ± 4.2
	125	106.0 ± 7.1	127.7 ± 10.3	161.0 ± 3.1	153.0 ± 6.1
	250	118.7 ± 8.1	119.7 ± 9.2	145.3 ± 11.9	166.3 ± 11.7
	500	99.7 ± 5.5	111.7 ± 6.2	137.3 ± 12.0	139.0 ± 9.5
	1,000	90.3 ± 2.8	106.0 ± 5.6	117.0 ± 8.0	109.7 ± 17.1[c]
	3,000	86.0 ± 2.3[c]	101.0 ± 7.2[c]	105.3 ± 1.7	122.7 ± 5.0[c]
	6,000	102.0 ± 11.2[c]	116.0 ± 12.5[c]	110.3 ± 16.4[c,d]	97.3 ± 9.6[c]
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		1,751.3 ± 68.7	1,984.0 ± 82.4	1,391.3 ± 37.1	1,178.0 ± 35.4

[a]Studies performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate served as the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[c]Precipitate on plate.
[d]Contamination.

Appendix B. Chemical Characterization and Dose Formulation Studies

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B.1. Procurement and Characterization of 2-Hydroxy-4-methoxybenzophenone

2-Hydroxy-4-methoxybenzophenone (2H4MBP) was obtained from Ivy Fine Chemicals Corporation (Cherry Hill, NJ) in one lot (20080801) that was used in the perinatal and 2-year studies. Identity and purity analyses were conducted under the analytical chemistry laboratory and study laboratory at Battelle (Columbus, OH). Reports on analyses performed in support of the 2H4MBP studies are on file at the National Institute of Environmental Health Sciences.

Lot 20080801 of the chemical, a light-yellow powder, was identified as 2H4MBP by infrared (IR) and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) with mass spectrometry (MS) detection. The IR spectrum was in good agreement with a reference spectrum[66] and the structure of 2H4MBP. Proton and carbon-13 NMR spectra were consistent with computer-predicted spectra and the structure of the test article. The mass spectrum of the major peak from the GC/MS analysis matched a reference spectrum[67] for 2H4MBP. A representative IR spectrum is presented in Table B-1 and Figure B-1.

The purity of lot 20080801 was determined using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection by system A and using GC with flame ionization detection (FID) by system B. Lot 20080801 was screened for common residual volatile solvents using GC system C with electron capture detection (ECD) and FID. Differential scanning calorimetry (DSC) was used to determine the purity of the test article using a PerkinElmer (Shelton, CT) Diamond differential scanning calorimeter scanning 1°C per minute over the range of 40°C to 75°C. In addition, Karl Fisher titration of lot 20080801 was performed by Galbraith Laboratories, Inc. (Knoxville, TN).

(A) For HPLC/UV analysis, the system included an Agilent 110 high-performance liquid chromatograph (Agilent Technologies, Inc., Santa Clara, CA) with UV detection (289 nm); a Synergi™ Fusion-RP column (100 mm × 3 mm, 2.5 µm particle size or 100 mm × 4.6 mm, 4 µm particle size; Phenomenex, Torrance, CA); and an isocratic mobile phase of acetonitrile:ASTM Type 1 water (40:60) at a flow rate of 0.8 mL/minute.

(B) For GC/FID analysis of initial bulk chemical purity, the system included an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) with FID; a Rtx-5, 30 m × 0.32 mm, 1.0 µm film thickness column (Restek, Bellefonte, PA), helium carrier gas at a flow rate of 3 mL/minute; and an oven temperature program of 80°C for 1 minute, then 20°C/minute to 200°C, then 10°C/minute to 280°C, held for 10 minutes.

(C) For GC analysis of residual volatiles in the test article, the system included an Agilent 6890 instrument (Agilent Technologies, Inc., Santa Clara, CA) with either ECD for halogenated volatiles or FID for non-halogenated volatiles; a Restek Rtx-624, 30 m × 0.53 mm, 3 µm film thickness column, helium carrier gas at a flow rate of 5 mL/minute; and an oven temperature program of 35°C for 14 minutes, then 15°C/minute to 40°C, held for 3 minutes, then 15°C/minute to 240°C, held for 2 minutes.

Purity assessment by HPLC/UV system A found no reportable impurities in lot 20080801. GC/FID analysis by system B yielded a purity of 99.8% and found one impurity with an area of 0.17% of the total peak area. No significant halogenated or nonhalogenated volatile impurities were found in the bulk chemical using GC/ECD or GC/FID by system C, respectively. Purity by DSC was 99.2%. Karl Fischer analysis indicated that no quantifiable water was present in the test article. The overall purity of lot 20080801 was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature, in sealed amber glass containers. Periodic reanalyses of the bulk chemical were performed during the perinatal and 2-year studies by the study laboratory using HPLC/UV by system A and no degradation of the bulk chemical was detected.

B.2. Preparation and Analysis of Dose Formulations

The dose formulations were prepared approximately monthly by mixing 2H4MBP with feed (Table B-1). Formulations were stored in sealed amber plastic bags at room temperature for up to 43 days.

Homogeneity studies of the 1,000 and 10,000 ppm dose formulations in NIH-07 and NTP-2000 feed were performed before the animal studies by the analytical chemistry and study laboratories with HPLC/UV by system A. Additional homogeneity studies of the 1,000 and 10,000 ppm dose formulations in NTP-2000 feed were performed during the chronic studies by the study laboratory with the same HPLC/UV system. Stability studies of the 1,000 ppm dose formulation in NIH-07 and NTP-2000 feed were performed by the analytical chemistry laboratory using the same analytical method. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed amber plastic bags at room temperature.

Periodic analyses of the dose formulations of 2H4MBP were conducted by the study laboratory using HPLC/UV system A. During the perinatal and 2-year studies, the dose formulations were analyzed 14 times; animal room samples were also analyzed (Table B-2; Table B-3). Of the dose formulations analyzed, all 65 for rats and all 67 for mice were within 10% of the target concentrations; all 18 animal room samples for rats and all 15 for mice were within 10% of the target concentrations.

Table B-1. Preparation and Storage of Dose Formulations in the Perinatal and Two-year Feed Studies of 2-Hydroxy-4-methoxybenzophenone

Rats	Mice
Preparation	
A premix of NIH-07 or NTP-2000 meal feed and 2-hydroxy-4-methoxybenzophenone was prepared in a Hobart processor, then layered into the remaining feed and blended in a Patterson Kelly twin-shell blender for approximately 15 minutes. The dose formulations were prepared approximately every 4 weeks.	Same as rats except that dose formulations were only prepared in NTP-2000 feed
Chemical Lot Number	
20080801	20080801
Maximum Storage Time	
42 days	43 days
Storage Conditions	
Stored in sealed amber plastic bags at room temperature	Stored in sealed amber plastic bags at room temperature
Study Laboratory	
Battelle (Columbus, OH)	Battelle (Columbus, OH)

Table B-2. Results of Analyses of Dose Formulations Administered to Rats in the Perinatal and Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
September 22, 2010	September 24, 2010	1,000	981	−2
		3,000	2,920	−3
		10,000	9,910	−1
	October 26, 2010[b]	1,000	945	−6
		3,000	2,740	−9
		10,000	9,550	−5
October 7, 2010	October 8, 2010	1,000	1,000	0
		1,000	989	−1
		3,000	3,020	1
		3,000	3,020	1
		10,000	10,000	0
		10,000	9,730	−3
	November 15, 2010[b]	1,000	950	−5

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
		3,000	2,840	−5
		10,000	9,450	−6
December 2, 2010	December 3, 2010	1,000	1,040	4
		1,000	1,030	3
		3,000	3,050	2
		3,000	3,070	2
		10,000	10,200	2
		10,000	10,100	1
January 26, 2011	January 26, 2011	1,000	998	0
		3,000	2,940	−2
		3,000	2,950	−2
		10,000	9,820	−2
	March 11, 2011[b]	1,000	971	−3
		3,000	3,020	+1
		10,000	9,550	−5
April 15, 2011	April 18, 2011	1,000	996	0
		1,000	992	−1
		3,000	2,970	−1
		3,000	2,960	−1
		10,000	9,860	−1
		10,000	9,890	−1
June 10, 2011	June 14, 2011	1,000	1,010	1
		1,000	1,010	1
		3,000	2,970	−1
		3,000	3,020	1
		10,000	9,890	−1
		10,000	9,790	−2
	July 25, 2011[b]	1,000	967	−3
		3,000	2,790	−7
		10,000	9,140	−9
September 7, 2011	September 9, 2011	1,000	1,020	2
		1,000	1,010	1

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
		3,000	2,910	−3
		3,000	2,960	−2
		10,000	9,830	−2
		10,000	9,730	−3
October 28, 2011	October 28, 2011	1,000	988	−1
		1,000	993	−1
		3,000	2,950	−2
		3,000	2,900	−3
		10,000	9,710	−3
		10,000	9,800	−2
January 20, 2012	January 20, 2012	1,000	1,010	1
		1,000	1,020	2
		3,000	2,960	−1
		3,000	2,930	−2
		10,000	9,750	−3
		10,000	9,730	−3
	March 6, 2012[b]	1,000	929	−7
		3,000	2,850	−5
		10,000	9,370	−6
March 16, 2012	March 16, 2012	1,000	1,030	3
		1,000	996	0
		3,000	3,020	1
		3,000	3,050	2
		10,000	9,960	0
		10,000	9,990	0
June 8, 2012	June 11, 2012	1,000	974	−3
		1,000	990	−1
		3,000	3,030	1
		3,000	2,950	−2
		10,000	9,960	0
		10,000	9,880	−1
August 31, 2012	August 31, 2012	1,000	984	−2

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
		3,000	3,010	0
		10,000	10,300	3
		10,000	10,200	2
	October 16, 2012[b]	1,000	974	−3
		3,000	2,860	−5
		10,000	9,600	−4

[a]Results of triplicate analyses.
[b]Animal room samples.

Table B-3. Results of Analyses of Dose Formulations Administered to Mice in the Two-year Feed Study of 2-Hydroxy-4-methoxybenzophenone

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
June 17, 2010	June 19, 2010	1,000	1,030	+3
		3,000	2,940	−2
		10,000	9,600	−4
	July 27, 2010[b]	1,000	951	−5
		3,000	2,770	−8
		10,000	9,120	−9
July 15, 2010	July 16, 2010	1,000	997	0
		1,000	993	−1
		3,000	2,920	−3
		3,000	2,930	−2
		10,000	9,770	−2
		10,000	9,770	−2
October 7, 2010	October 8, 2010	1,000	1,000	0
		1,000	989	−1
		3,000	3,020	1
		3,000	3,020	1
		10,000	10,000	0
		10,000	9,730	−3
December 2, 2010	December 3, 2010	1,000	1,040	4

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
		1,000	1,030	3
		3,000	3,050	2
		3,000	3,070	2
		10,000	10,200	2
		10,000	10,100	1
	January 13, 2011[b]	1,000	943	−6
		3,000	2,760	−8
		10,000	9,260	−7
January 26, 2011	January 26, 2011	1,000	998	0
		3,000	2,940	−2
		3,000	2,950	−2
		10,000	9,820	−2
	March 11, 2011[b]	1,000	977	−2
		3,000	2,910	−3
		10,000	9,920	−1
April 15, 2011	April 18, 2011	1,000	996	0
		1,000	992	−1
		3,000	2,970	−1
		3,000	2,960	−1
		10,000	9,860	−1
		10,000	9,890	−1
June 10, 2011	June 14, 2011	1,000	1,010	1
		1,000	1,010	1
		3,000	2,970	−1
		3,000	3,020	1
		10,000	9,890	−1
		10,000	9,790	−2
	July 25, 2011[b]	1,000	967	−3
		3,000	2,870	−4
		10,000	9,560	−4

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
September 7, 2011	September 9, 2011	1,000	1,020	2
		1,000	1,010	1
		3,000	2,910	−3
		3,000	2,960	−2
		10,000	9,830	−2
		10,000	9,730	−3
October 28, 2011	October 28, 2011	1,000	988	−1
		1,000	993	−1
		3,000	2,950	−2
		3,000	2,900	−3
		10,000	9,710	−3
		10,000	9,800	−2
January 20, 2012	January 20, 2012	1,000	1,010	1
		1,000	1,020	2
		3,000	2,960	−1
		3,000	2,930	−2
		10,000	9,750	−3
		10,000	9,730	−3
	March 6, 2012[b]	1,000	975	−3
		3,000	2,840	−5
		10,000	9,360	−6
March 16, 2012	March 16, 2012	1,000	1,030	3
		1,000	996	0
		3,000	3,020	1
		3,000	3,050	2
		10,000	9,960	0
		10,000	9,990	0
June 8, 2012	June 11, 2012	1,000	974	−3
		1,000	990	−1
		3,000	3,030	1

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
		3,000	2,950	−2
		10,000	9,960	0
		10,000	9,880	−1

[a]Results of triplicate analyses.
[b]Animal room samples.

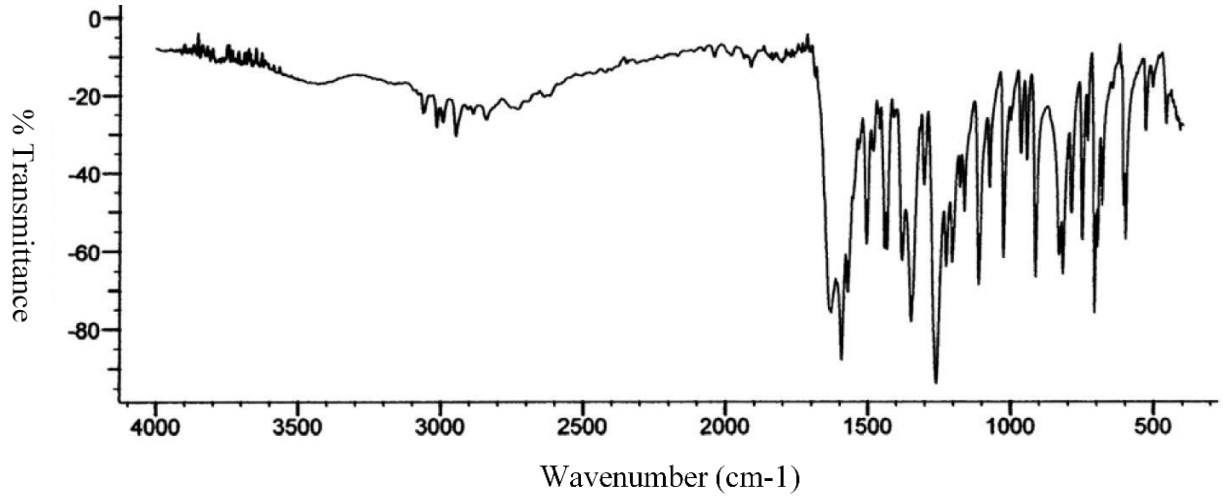

Figure B-1. Infrared Absorption Spectrum of 2-Hydroxy-4-methoxybenzophenone

Appendix C. Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration

Tables

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Table C-1. Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	22.26
Ground #2 Yellow Shelled Corn	22.18
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	5.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table C-2. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
Vitamin A	4,000 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	1,000 IU	D-activated animal sterol
Vitamin K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	–
α-Pantothenic Acid	10 mg	α-Calcium pantothenate
Riboflavin	3.3 mg	–
Thiamine	4 mg	Thiamine mononitrate
B12	52 μg	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	α-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished product.

Table C-3. Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	14.8 ± 0.516	14.2–16.8	28
Crude Fat (% by weight)	8.69 ± 0.357	8.0–9.7	28
Crude Fiber (% by weight)	9.47 ± 0.594	8.34–11.6	28
Ash (% by weight)	5.25 ± 1.76	4.6–14.2	28

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Amino Acids (% of Total Diet)			
Arginine	0.802 ± 0.075	0.67–0.97	28
Cystine	0.220 ± 0.022	0.15–0.25	28
Glycine	0.703 ± 0.038	0.62–0.80	28
Histidine	0.342 ± 0.071	0.27–0.68	28
Isoleucine	0.549 ± 0.041	0.43–0.66	28
Leucine	1.097 ± 0.064	0.96–1.24	28
Lysine	0.700 ± 0.106	0.31–0.86	28
Methionine	0.410 ± 0.042	0.26–0.49	28
Phenylalanine	0.623 ± 0.047	0.47–0.72	28
Threonine	0.512 ± 0.042	0.43–0.61	28
Tryptophan	0.155 ± 0.027	0.11–0.20	28
Tyrosine	0.420 ± 0.066	0.28–0.54	28
Valine	0.666 ± 0.040	0.55–0.73	28
Essential Fatty Acids (% of Total Diet)			
Linoleic	3.95 ± 0.234	3.49–4.55	28
Linolenic	0.31 ± 0.031	0.021–0.368	28
Vitamins			
Vitamin A (IU/kg)	378 ± 79.35	203–529	28
Vitamin D (IU/kg)	1,000[a]	–	–
α-Tocopherol (ppm)	2,543 ± 13,044	27.0–69,100	28
Thiamine (ppm)b	8.39 ± 1.87	3.9–12.5	28
Riboflavin (ppm)	8.06 ± 2.83	4.20–17.50	28
Niacin (ppm)	78.6 ± 8.26	66.4–98.2	28
Pantothenic Acid (ppm)	26.6 ± 11.22	17.4–81.0	28
Pyridoxine (ppm)b	9.78 ± 2.08	6.44–14.3	28
Folic Acid (ppm)	1.58 ± 0.44	1.15–3.27	28
Biotin (ppm)	0.32 ± 0.09	0.20–0.704	28
B12 (ppb)	50.6 ± 35.5	18.3–174.0	28
Choline (as Chloride) (ppm)	2,615 ± 635	1,160–3,790	28

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Minerals			
Calcium (%)	0.913 ± 0.046	0.831–1.03	28
Phosphorus (%)	0.550 ± 0.099	0.053–0.613	28
Potassium (%)	0.667 ± 0.030	0.626–0.733	28
Chloride (%)	0.393 ± 0.045	0.300–0.517	28
Sodium (%)	0.197 ± 0.026	0.160–0.283	28
Magnesium (%)	0.217 ± 0.055	0.185–0.490	28
Sulfur (%)	0.170 ± 0.029	0.116–0.209	14
Iron (ppm)	191.6 ± 36.8	135–311	28
Manganese (ppm)	50.1 ± 9.59	21.0–73.1	28
Zinc (ppm)	57.4 ± 26.0	23.3–184.0	28
Copper (ppm)	7.53 ± 2.53	3.21–16.3	28
Iodine (ppm)	0.531 ± 0.201	0.158–0.972	28
Chromium (ppm)	0.916 ± 0.908	0.330–3.97	27
Cobalt (ppm)	0.225 ± 0.154	0.086–0.964	26

[a]From formulation.
[b]As hydrochloride.

Table C-4. Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.223 ± 0.063	0.149–0.385	28
Cadmium (ppm)	0.053 ± 0.012	0.038–0.094	28
Lead (ppm)	0.138 ± 0.108	0.064–0.474	28
Mercury (ppm)[a]	0.014 ± 0.008	0.01–0.049	28
Selenium (ppm)	0.162 ± 0.030	0.029–0.209	28
Aflatoxins (ppb)[a]	5 ± 0.00	5.0–5.0	28
Nitrate Nitrogen (ppm)[b]	15.62 ± 6.125	10.0–35.1	28
Nitrite Nitrogen (ppm)[a,b]	0.61 ± 0.002	0.60–0.61	28
BHA (ppm)[a,c]	1 ± 0.00	1.0–1.0	28
BHT (ppm)[a,c]	1.08 ± 0.40	1.0–3.14	28

	Mean ± Standard Deviation[b]	Range	Number of Samples
Aerobic Plate Count (CFU/gm)	10 ± 0.00	10.0–10.0	28
Coliform (MPN/gm)	3.0 ± 0.00	3.0–3.0	28
E. coli (MPN/gm)[a]	10 ± 0.00	10.0–10.0	28
Salmonella (MPN/gm)	Negative	–	–
Total Nitrosamines (ppb)[d]	11.1 ± 5.55	3.2–24.5	28
N-Ndimethylamine (ppb)[d]	2.5 ± 1.6	1.0–6.8	28
N-Npyrrolidine (ppb)[d]	8.7 ± 5.1	2.1–20.0	28
Pesticides (ppm)			
α-BHC[a]	<0.01	–	28
β-BHC[a]	<0.02	–	28
γ-BHC[a]	<0.01	–	28
δ-BHC[a]	<0.01	–	28
Heptachlor[a]	<0.01	–	28
Aldrin[a]	<0.01	–	28
Heptachlor Epoxide[a]	<0.01	–	28
DDE[a]	<0.01	–	28
DDD[a]	<0.01	–	28
DDT[a]	<0.01	–	28
HCB[a]	<0.01	–	28
Mirex[a]	<0.01	–	28
Methoxychlor[a]	<0.05	–	28
Dieldrin[a]	<0.01	–	28
Endrin[a]	<0.01	–	28
Telodrin[a]	<0.01	–	28
Chlordane[a]	<0.05	–	28
Toxaphene[a]	<0.10	–	28
Estimated PCBs[a]	<0.20	–	28
Ronnel[a]	<0.01	–	28
Ethion[a]	<0.02	–	28
Trithion[a]	<0.05	–	28
Diazinon[a]	<0.10	–	28

	Mean ± Standard Deviation[b]	Range	Number of Samples
Methyl Chlorpyrifos	0.09 ± 0.073	0.02–0.315	28
Methyl Parathion[a]	<0.02	–	28
Ethyl Parathion[a]	<0.02	–	28
Malathion	0.1 ± 0.093	0.02–0.355	28
Endosulfan I[a]	<0.01	–	28
Endosulfan II[a]	<0.01	–	28
Endosulfane Sulfate[a]	<0.03	–	28

CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; PCB = polychlorinated biphenyl.
[a]All values were less than the detection limit. The detection limit is given as the mean.
[b]Sources of contamination include alfalfa, grains, and fish meal.
[c]Sources of contamination include soy oil and fish meal.
[d]All values were corrected for percent recovery.

Appendix D. Sentinel Animal Program

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D.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or exposed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For these toxicology and carcinogenesis studies, blood samples were collected from the rodents and allowed to clot, and the serum was separated. Additionally, fecal samples were collected and tested for *Helicobacter* species. All samples were processed appropriately with serology testing and sent to IDEXX BioResearch (formerly Rodent Animal Diagnostic Laboratory [RADIL], University of Missouri), Columbia, MO, for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected per the following:

- Rats: 10 females at end of quarantine, and 4 weeks postarrival; 5 animals per sex at 6, 15, and 18 months, and study termination
- Mice: 5 animals per sex per time period

D.2. Results

Rats: Positive for pinworms (*Syphacia spp*.) All other test results were negative. Mice: All test results were negative.

Table D-1. Methods and Results for Sentinel Animal Testing

Method and Test	Time of Collection
Rats	
Multiplex Fluorescent Immunoassay	
Kilham Rat Virus	End of quarantine[a]; 4 weeks postarrival[b]; 6, 15, and 18 months; stud termination
Mycoplasma Pulmonis	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Pneumonia Virus of Mice	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Rat Coronavirus/Sialodacryoadenitis Virus	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Rat Minute Virus	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination

Method and Test	Time of Collection
Rat Parvo Virus	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Rat Theilovirus	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Sendai	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Toolan's H-1	End of quarantine; 4 weeks postarrival; 6, 15, and 18 months; study termination
Immunofluorescence Assay	
Pneumocystis carinii	6 months
Mice	
Multiplex Fluorescent Immunoassay	
Ectromelia Virus	6 weeks postarrival; 6, 12, and 18 months; study termination
Epizootic Diarrhea of Infant Mice	6 weeks postarrival; 6, 12, and 18 months; study termination
Lymphocytic Choriomeningitis Virus	6 weeks postarrival; 6, 12, and 18 months; study termination
Mycoplasma Pulmonis	6 weeks postarrival; 6, 12, and 18 months; study termination
Mouse Hepatitis Virus	6 weeks postarrival; 6, 12, and 18 months; study termination
Mouse Norovirus	6 weeks postarrival; 6, 12, and 18 months; study termination
Mouse Parvovirus	6 weeks postarrival; 6, 12, and 18 months; study termination
Minute Virus of Mice	6 weeks postarrival; 6, 12, and 18 months; study termination
Pneumonia Virus of Mice	6 weeks postarrival; 6, 12, and 18 months; study termination
Reovirus	6 weeks postarrival; 6, 12, and 18 months; study termination
Sendai	6 weeks postarrival; 6, 12, and 18 months; study termination
Theiler's Murine Encephalomyelitis Virus GDVII	6 weeks postarrival; 6, 12, and 18 months; study termination
Immunofluorescence Assay	
Ectromelia Virus	6 months
Epizootic Diarrhea of Infant Mice	6 weeks postarrival; 6 months
Mouse Hepatitis Virus	Study termination
Mouse Norovirus	6 and 12 months
Polymerase Chain Reaction	
Helicobacter species	18 months

[a]Age-matched nonpregnant females.
[b]Time-mated females that did not have a litter.

Appendix E. Microarray Analysis

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E.1. Objective

The objective of the microarray study was to evaluate the transcriptional changes in liver from rats exposed to 0 or 10,000 ppm 2H4MBP. At the 14-week interim evaluation, livers were analyzed from male Sprague Dawley rats.

E.2. Methods

E.2.1. RNA Isolation, cDNA Synthesis, and Array Hybridization

Liver tissues were excised from five vehicle control F_1 male rats and five 10,000 ppm F_1 male rats from the 14-week interim evaluation performed as a part of the 2-year carcinogenicity study. Tissues were immediately frozen in liquid nitrogen at collection and transported to the Battelle Biomedical Research Center (Columbus, OH). The liver tissues were removed and added to lysis buffer, and each sample was then homogenized using OmniTip™ plastic disposable probes (Omni International, Marietta, GA). Following homogenization samples were centrifuged and the RNA was extracted from the supernatant using the Qiagen RNeasy Midi Kit (Qiagen, Valencia, CA). RNA concentration and purity were determined by UV analysis using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples were assessed for RNA integrity using an RNA 6000 Nano Chip kit with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Total RNA (100 ng) was used to synthesize single-stranded DNA, which was subsequently converted into a double stranded eDNA template for transcription. An in vitro transcription (IVT) reaction, which incorporates biotinylated ribonucleotide analogs, was then used to create labeled amplified RNA (aRNA). This RNA target preparation was performed using the Affymetrix GeneChip® 3' IVT Express Kit (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's protocol. All incubation steps during this preparation were completed using an Eppendorf Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany). Labeled aRNA was fragmented and hybridized to the Affymetrix Rat Genome 230 2.0 Array (31,099 probe sets) using an Affymetrix GeneChip Hybridization Oven 640. Washing and staining of the arrays were completed using the Affymetrix GeneChip Hybridization Wash and Stain kit and performed using the Fluidics Station 450 according to the Affymetrix recommended protocol (FS450_0001). After washing and staining, the arrays were scanned using an Affymetrix GeneChip Scanner 3000 7G and the raw microarray data (.CEL files) were acquired using Affymetrix GeneChip Command Console® Software (AGCC).

E.2.2. Microarray Analysis

E.2.2.1. Analysis of GeneChip Data Quality

Quality control (QC) measurements from each Affymetrix GeneChip array were evaluated to determine the quality of the microarray data generated. Affymetrix recommended guidelines for evaluating quality were used to evaluate the output files for each GeneChip array using the R/Bioconductor package, Simpleaffy.[117] The following QC parameters were evaluated for each chip: average background, scale factor, percent of genes scored as present, 3' to 5' ratios for the internal control genes beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), values for hybridization control transcripts, and values for poly (A) controls. In addition, the data were visually assessed outliers using intensity distribution histograms and principal component

analysis. These assessments contributed to the determination that the quality of the GeneChip array data obtained from all 10 RNA samples is sufficient to proceed with statistical analysis and biological interpretation.

E.2.2.2. Statistical Analysis

The 10 .CEL files representing the 10 liver samples from the study were normalized using the robust multiarray algorithm (RMA)[118] using Patek Genomics Suite v 6.6 (Partek, Inc, St. Louis, MO) using the commented settings in the software. Probe sets showing differential expression between the two dose groups were identified using an ANOVA ($p < 0.05$) combined with a fold change of >|1.5|.

E.2.2.3. Biological Interpretation

Ingenuity Pathway Analysis™ (IPA) (Ingenuity Systems®, Inc., Redwood City, CA) was used to facilitate biological interpretation of the transcriptional changes produced by 2H4MBP treatment.

The IPA Core Analysis was performed on June 11, 2019. Enrichment and activation analysis was carried out for IPA disease/biological/toxicity functions. In addition, an IPA Upstream Analysis was performed. Enrichment p values were calculated using a right-tailed Fisher's Exact Test combined with a Benjamini-Hochberg method of multiple testing correction. The p value ($p < 0.05$) was determined by how many differentially expressed genes (DEGs) overlapped that are annotated into gene groups (e.g., canonical pathways, biological functions).

Activation/inhibition analysis was performed using the annotation in the IPA Knowledge Base. To determine if there was plausible activation/inhibition of disease/biological/toxicity functions, transcription factors, and chemical signaling signatures, a Z-score was calculated. The Z-score was determined by concordance of observed patterns of regulation (up or down) with known effects on biological functions or effects of transcription factors/chemicals (activation or inhibition of target genes) as annotated in the Ingenuity Knowledge Base. A gene set was deemed to be activated (Z-score >2), inhibited (Z-score <−2), or not affected on the basis of the Z-score.

E.3. Results

E.3.1. Differential Gene Expression

Whole-rat genome Affymetrix 230 2.0 microarrays were used to assess the effect of 10,000 ppm of 2H4MBP in the diet for 14 weeks on male Sprague Dawley rat liver. Treatment with 2H4MBP led to the differential expression of 357 probe sets (67 up-regulated, 290 down-regulated) that mapped to 273 unique genes. Overall the effect on the transcriptome was deemed to be weak relative to high-dose studies that are typical of most toxicogenomics assessments. Notably *Cyp2b1/2*, *Cyp1a1*, and *Cyp4a1* were all moderately up-regulated along with other biotransformation-related genes such as *Gsta2*, *Aldh1a7*, and *Abcc3*. Up-regulation of these genes suggests the liver was adapting to the presence of chemical stressor by up-regulating metabolic pathways to facilitate clearance. The top five up- and down-regulated probe sets as determined by fold change are shown in Table E-1.

Five hundred twenty-seven IPA disease/biological/toxicity functions showed significant enrichment, however only a small set were also determined to be activated or inhibited by 2H4MBP treatment. Those that were activated include hepatic steatosis, hypoplasia of organ, and G2 phase. Several functions, all primarily related to cell cycle and growth, were inhibited by 2H4MBP treatment. The enriched and activated/inhibited functions are listed in Table E-2.

Twenty-six IPA canonical pathways showed significant enrichment following 2H4MBP treatment. Of the 26 pathways, only a small subset showed inhibition and all were related to cell cycle/proliferation. The enriched and activated/inhibited pathways are listed in Table E-3.

The IPA Upstream Analysis identified >700 enriched regulatory agents, however, only 32 showed patterns that were suggestive of activation of inhibition. A sizable number of the regulatory agents were prototype chemicals that effect specific processes (e.g., PXR activation) and lead to specific patterns of transcriptional response (i.e., signatures). Identification of these patterns in the 2H4MBP transcriptional response data suggests that 2H4MBP may be interacting with the same biological targets as the prototype agents. Caution must be used when interpreting these results because genes are often shared across the signatures, hence differentiating between highly redundant upstream regulatory processes that are driving transcriptomic changes is challenging.

Findings for 2H4MBP are suggestive of effects on glucocorticoid (dexamethasone) and vitamin D (calcitriol) signaling and potentially on estrogen signaling (raloxifene). Other potential regulatory features in the 2H4MBP signaling cascade include miR-30c-5p and let-7 microRNAs and some inhibitory effects on cytokine signaling (i.e., TNF, IL1β, and IFNβ1). Notably, there is a suggestion that estrogen receptor (*Esr1*) signaling was inhibited by 2H4MBP, although an effect on *Esr1* is unlikely based on additional analysis that was performed (see the Other Notable Findings section in this appendix, below). The enriched and activated/inhibited pathways are listed in Table E-4.

E.3.2. Other Notable Findings

Due to the potential estrogenic properties of 2H4MBP, a specific analysis of estrogen response genes was performed. A subset of genes is known to be strongly up-regulated in male rat liver by pharmacological estrogens. The up-regulation of these genes in response to estrogens has been documented in both the DrugMatrix and TG-Gates data sets,[119; 120] which are accessible through the Illumina Correlation Engine.[121] The up-regulated genes include *Rbp7*, *Lifr*, *Cited4*, *Ksr1*, and *Ctr9*. None of these genes was identified as differentially expressed in the liver of male rats following 2H4MBP exposure. This observation suggests that it is unlikely that 2H4MBP produced a systemic estrogenic effect within the context of the 14-week study.

E.4. Conclusions

The overall transcriptomic response of rat liver to 14 weeks of exposure to 10,000 ppm 2H4MBP was weak. This is consistent with the absence of pathological findings in the liver following 2 years of exposure at this dose. The biological interpretation suggests there may be marginal, nonspecific effects on steroid hormone homeostasis that may be related findings in the adrenal cortex, testis, uterus, and ovary in the 2-year study. Notably, these effects do not appear to be related to direct effects on the estrogen receptor as opposed to other potential mechanisms that

have yet to be characterized. One generally consistent observation was the inhibition of cell cycle-related processes, which could potentially be related to the decreased body weight observed in the 10,000 ppm groups from the 14-week interim evaluation. Finally, the weak induction of some of the nuclear receptor regulated P450s (e.g., *Cyp2b1/2*) are likely associated with microsomal enzyme induction and the observed liver weight increase at 10,000 ppm 2H4MBP.

Table E-1. Top Five Up- and Down-regulated Probesets in the Liver of Male Sprague Dawley Rats Administered 10,000 ppm 2H4MBP in Feed for 14 Weeks

Probeset ID	Gene Symbol	Entrez Gene	Fold Change
Up-regulated			
1368718_at	Aldh1a7	29651	21.9409
1378249_x_at	LOC102554740 /// LOC103693750	102554740 /// 103693750	2.83937
1371076_at	*Cyp2b1 /// Cyp2b2*	24300 /// 361523	2.63343
1368321_at	Egr1	24330	2.43895
1394541_at	Unknown	Unknown	2.37995
Down-regulated			
1390317_at	RGD1561849	500393	−2.6521
1369415_at	Bhlhe40	79431	−3.11306
1395255_at	Onecut1	25231	−3.16945
1387760_a_at	Onecut1	25231	−3.77045
1371034_at	Onecut1	25231	−3.98444

Table E-2. Ingenuity Pathway Analysis Enriched Disease and Biological/Toxicity Functions that Exhibit Activation/Inhibition in the Liver of Male Sprague Dawley Rats Administered 10,000 ppm 2H4MBP in Feed for 14 Weeks

Diseases or Biological/Toxicity Functions	Enrichment P Value	Activation Z-Score	Predicted Activation State
Hepatic Steatosis	0.00116	2.327	Activate
Hypoplasia of Organ	0.00299	2.138	Activate
G2 Phase	0.00229	2	Activate
Increased Levels of Red Blood Cells	0.00139	−2	Inhibited
S Phase	0.00183	−2.01	Inhibited
Vasculogenesis	0.00665	−2.092	Inhibited
Mitosis	0.00373	−2.098	Inhibited
Formation of Gonadal Cells	0.00475	−2.169	Inhibited

Diseases or Biological/Toxicity Functions	Enrichment P Value	Activation Z-Score	Predicted Activation State
Maturation of Myeloid Cells	0.00096	−2.181	Inhibited
Development of Genitourinary System	0.00114	−2.189	Inhibited
Angiogenesis	0.00172	−2.221	Inhibited
Development of Vasculature	0.00178	−2.223	Inhibited
Hyperplasia of Exocrine Gland	0.00395	−2.236	Inhibited
Genitourinary Tumor	0.00313	−2.358	Inhibited
Cell Cycle Progression	$6.54E^{-06}$	−2.376	Inhibited
Development of Genital Organ	0.000931	−2.376	Inhibited
Transactivation of RNA	0.00258	−2.399	Inhibited
Cell Proliferation of Tumor Cell Lines	0.00153	−2.595	Inhibited
Development of Reproductive System	0.000378	−2.91	Inhibited
Growth of Organism	0.00696	−3.468	Inhibited

Table E-3. Ingenuity Pathway Analysis Enriched Canonical Pathways that Exhibit Activation/Inhibition in the Liver of Male Sprague Dawley Rats Administered 10,000 ppm 2H4MBP in Feed for 14 Weeks

Ingenuity Canonical Pathways	Enrichment P Value	Activation Z-Score	Predicted Activation State
Acute Myeloid Leukemia Signaling	0.0355	−2.236	Inhibition
ERK5 Signaling	0.0372	−2	Inhibition
Melanocyte Development and Pigmentation Signaling	0.0380	−2.236	Inhibition
ErbB2–ErbB3 Signaling	0.0490	−2	Inhibition
Mouse Embryonic Stem Cell Pluripotency	0.0490	−2.236	Inhibition

Table E-4. Ingenuity Pathway Analysis Enriched Upstream Regulators that Exhibit Activation/Inhibition in the Liver of Male Sprague Dawley Rats Administered 10,000 ppm 2H4MBP in Feed for 14 Weeks

Upstream Regulator	Molecule Type	Enrichment P Value	Activation Z-Score	Predicted Activation State
MiR-30c-5p (and Other MiRNAs w/ Seed GUAAACA)	Mature microRNA	0.000422	2.828	Activated

Upstream Regulator	Molecule Type	Enrichment P Value	Activation Z-Score	Predicted Activation State
Let-7	MicroRNA	0.019	2.763	Activated
U0126	Chemical – kinase inhibitor	0.0368	2.314	Activated
Sulindac Sulfide	Chemical drug	1.92E−05	2.209	Activated
Calcitriol	Chemical drug	0.0411	2.173	Activated
Dexamethasone	Chemical drug	0.000916	2.096	Activated
LY294002	Chemical – kinase inhibitor	0.00632	2.058	Activated
Cigarette Smoke	Chemical toxicant	0.00578	2.007	Activated
ZBED6	Transcription regulator	0.000273	2	Activated
Ursolic Acid	Chemical drug	0.00175	2	Activated
Salirasib	Chemical drug	0.00961	2	Activated
MiR-199a-5p (and Other MiRNAs w/ Seed CCAGUGU)	Mature microRNA	0.0245	2	Activated
EHHADH	Enzyme	0.000111	−2	Inhibited
HSD17B4	Enzyme	0.000718	−2	Inhibited
NORAD	Other	0.0027	−2	Inhibited
Esr1	Ligand-dependent nuclear receptor	0.0136	−2.04	Inhibited
Lipopolysaccharide	Chemical drug	0.000845	−2.12	Inhibited
Tgf Beta	Group	0.0234	−2.126	Inhibited
IFNB1	Cytokine	0.036	−2.162	Inhibited
Bucladesine	Chemical toxicant	0.0481	−2.19	Inhibited
E2F1	Transcription regulator	0.0453	−2.191	Inhibited
TGFB2	Growth factor	0.00702	−2.205	Inhibited
CSHL1	Growth factor	0.00302	−2.207	Inhibited
E2F3	Transcription regulator	0.0292	−2.219	Inhibited
Tributyrin	Chemical drug	0.00418	−2.236	Inhibited
Mycophenolic Acid	Chemical drug	0.00949	−2.236	Inhibited
IL1B	Cytokine	0.00709	−2.36	Inhibited
PDGF BB	Complex	0.00181	−2.381	Inhibited
Raloxifene	Chemical drug	0.0272	−2.433	Inhibited
Insulin	Group	0.000964	−2.446	Inhibited
TNF	Cytokine	0.00658	−2.672	Inhibited
HGF	Growth factor	0.0154	−2.745	Inhibited

Appendix F. Endocrine Disruptor Screening Panel Studies

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F.1. Competitive Estrogen Receptor Binding Assay

F.1.1. Methodology

All procedures were conducted in accordance with the U.S. Environmental Protection Agency (EPA) guideline Office of Prevention, Pesticides and Toxic Substances (OPPTS) 890.1250.[40]

Briefly, 7-day postovariectomy uterine tissue from 12- to 13-week old Sprague Dawley rats was procured from Harlan Laboratories (now Envigo, Indianapolis, IN) and shipped overnight on dry ice. Uterine tissue was inspected for signs of residual ovarian tissue, and, if present, that tissue was discarded. Cytosolic fractions were prepared, pooled, and either used immediately or frozen at −80°F. If frozen, once subsequently thawed, unused aliquots were discarded. Three saturation bindings assays to support each run of the respective sunscreen ingredient were performed according to OPPTS 890.1250 and were shown to be acceptable.[40]

Radioactivity was assessed via a scintillation counter (PerkinElmer Tri-Carb 2910TR Liquid Scintillation Analyzer Model B2910) and each vial was counted for at least one minute with quench correction for determination of disintegrations per minute (DPM) per vial. Standards (^{3}H, ^{14}C, and background) were used to verify accurate counting. For competitive binding, 1 nM of [^{3}H]-17β-estradiol was prepared on the day of assay and the specific activity was adjusted for decay over time prior to performing dilutions. The concentration of estrogen receptor (ER) was sufficient to bind 10–15% of the radioligand, and the incubation time was 16–20 hours at 4°C. The competitive binding assay was determined to be functioning correctly on the basis of criteria set forth in OPPTS 890.1250; specifically, increasing concentrations of unlabeled 17β-estradiol displaced [^{3}H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding.[40]

The curve fit to the radio-inert estradiol data points using nonlinear regression descended from 90% to 10% over approximately an 81-fold increase in the concentration of the test chemicals. Ligand depletion was minimal—the ratio of total binding in the absence of competitor to the total amount of [^{3}H]-17β-estradiol added per assay tube was no greater than 15%. The parameter values (Top, Bottom, and Hill slope) for 17β-estradiol and 19-norethindrone (positive control) were within the tolerance bounds outlined in the OPPTS guideline.[40] The dimethyl sulfoxide (DMSO) solvent control did not alter the sensitivity or reliability of the assay—the acceptable limit of ethanol concentration in the assay tube—was 3%, the acceptable limit of DMSO concentration was ≤10%, and all tubes contained the same amount of solvent. The negative control substance, octyltriethoxysilane, did not displace more than 25% of the radioligand from the ER on average across all concentrations.

The sunscreen ingredients were assessed over a concentration range that fully defined the top of the curve (i.e., a range that showed that a top plateau was achieved) and the top was within 25% of either the solvent control or the value for the lowest concentration of the 17β-estradiol l standard for that run. The classification of a chemical as a binder or nonbinder was made on the basis of the average results of three nonconcurrent runs, each of which met the performance criteria and, taken together, were consistent with each other, as per OPPTS guideline 890.1250.[40] Each run was classified as “interactive,” “not interactive,” “equivocal,” or “equivocal up to the limit of the concentrations tested.” A run was classified as “interactive” with the ERs if the lowest point on the fitted response curve within the range of the data was less than 50%.

"Percent" (%) refers to binding of the radiolabeled estradiol. Thus, "less than 50%" means that less than 50% of the radiolabeled estradiol was bound, or, equivalently, that more than 50% of the radiolabeled estradiol had been displaced from the receptor. A run was classified as "equivocal up to the limit of concentrations tested" if there were no data points at or above sunscreen ingredient concentration of 10^{-6} M and one of the two following conditions held: (1) a binding curve could be fit but 50% or less of the radiolabeled estradiol was displaced by a concentration of 10^{-6} M; or (2) a binding curve could not be fit and the lowest average percent binding among the concentration groups in the data was above 50%. A run was classified as "not interactive" if there were usable data points at or above 10^{-6} M and either: (1) the lowest point on the fitted response curve within the range of the data was above 75%; or (2) a binding curve could not be fit and the lowest average percent binding among the concentration groups in the data was above 75%. A run was classified as "equivocal" if it fell in none of these categories.

After each run was classified, the chemical was classified by assigning the following values to each run and averaging across runs: interactive = 2, equivocal = 1, and not interactive = 0. The chemical classification, based on the average of all the runs performed for a chemical, was calculated as: interactive (average ≥ 1.5), equivocal (0.5 ≤ average < 1.5), and not interactive (average < 0.5). Data points were plotted using GraphPad curve fit for:

$$\mathbf{IC_{50}}\text{: } Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1 + 10\text{^}((\log IC_{50}-X)*\text{Hillslope} + \log((\text{Top}-\text{Bottom})/(50-\text{Bottom}) - 1))).$$

The Bottom was constrained to >0 for 2H4MBP and 2-Ethylhexyl p-methoxycinnamate (EHMC).

F.1.2. Results

In each of the three independent ER binding experiments, the maximal mean specific binding was >75% at every soluble 2H4MBP and EHMC concentration assessed, categorizing them as "not interactive" (Figure F-1). When the specific binding was averaged using the scoring system as described in the OPPTS guideline, 2H4MBP and EHMC were classified as "not interactive."[40]

The half maximal inhibitory concentration (IC_{50}), $\log IC_{50}$, Top, Bottom, and Hill slopes of the curves were determined using the Hill model for 2H4MBP, EHMC and the reference agents (Table F-1). In general, the sunscreen ingredient-specific binding data could be fit to IC_{50} models but were often ascribed a fit of "ambiguous" by the software, likely due to the weakness of response. The IC_{50}s for the "not interactive" sunscreen ingredients, 2H4MBP and EHMC, ranged from approximately 2.3×10^{-4} to 14.8×10^{-4} M and approximately 1.9×10^{-3} to 4.6×10^{4} M, respectively.

The weak positive control 19-norethindrone exhibited a $\log IC_{50}$ of approximately −5.5 to −5.6 M, whereas 17β-estradiol displayed a $\log IC_{50}$ of approximately −8.8 to −9.0 M. The Hill slope for these reference agents was between approximately −0.7 and −1.1.

F.2. Estrogen Receptor Transcriptional Activation in a Human Cell Line (HeLa-9903)

F.2.1. Methodology

All procedures were conducted in accordance with EPA guideline OPPTS 890.1300.[41] Briefly, the stably transfected hERα-HeLa-9903 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). This cell line is derived from a human cervical tumor and has two stably inserted constructs—the hERα expression construct (encoding the full-length human receptor) and a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin estrogen-responsive element driven by a mouse metallothionein (MT) promoter TATA element. Consequently, the hERα-HeLa-9903 cell line can measure the ability of a test substance to induce hERα-mediated transactivation of luciferase gene expression and consequently can be used to assess the ability of a test substance to act as an agonist of hERα.

The cell line was certified to be free of mycoplasma, was passage 21 for the assay, and was maintained on phenol-red free media. The functional stability of the cell line was monitored using the following reference chemicals: 17β-estradiol, 17α-estradiol, 17α-methyltestosterone, and corticosterone. A complete concentration response curve for each reference compound was run each time the transcriptional activation assay was performed and the $logPC_{50}$, $logPC_{10}$, $logEC_{50}$, Hill slope, and fold induction values calculated, compared, and deemed consistent with guideline acceptable range values.

Cell viability was monitored by a two-read propidium iodide uptake assay using a Packard Fusion fluorescence plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. The first read was taken 24 hours after the addition of the control and test substances. The measured fluorescence indicates spontaneous cell death and control/test material induced cytotoxicity. The cells were then lysed, and a second read was taken, indicating 100% cell death. The first read is then subtracted from the second read and the results of the subtracted reads are directly proportional to the viability of the cells. As a positive control for inducing cell death, 125 μM digitonin was used. Dose groups were normalized to vehicle control to generate percent cell viability.

DMSO was selected as a suitable vehicle for 2H4MBP and EHMC. 2H4MBP and EHMC solutions up to $10^{-4.5}$ M (the limit concentration for the assay) can be prepared while limiting the final concentration of DMSO in the assay medium to 0.1% (v/v). 17α-methyltestosterone,

17α-estradiol, corticosterone, and 17β-estradiol. The limit of solubility was determined by visual inspection of the test materials and controls after preparation of the final 1x dosing solutions in culture media. A sample of the 1x dosing solution was placed into wells of a clear 96-well plate and an endoscope was used to assess precipitation in each sample. Cytotoxicity and test article precipitation were assessed at $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$, 10^{-4}, $10^{-3.5}$, and 10^{-3} M. If a dose level of sunscreen ingredient produced cytotoxicity or precipitation, that dose level was not used. Cytotoxicity was observed at 10^{-3} M 2H4MBP or EHMC. The final concentrations of 2H4MBP and EHMC tested in replicate in the ER transcriptional activation assay were 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. Data points were plotted using GraphPad curve fit for:

EC_{50}: Y = Bottom + (Top−Bottom)/(1 + 10^((logIC_{50}−X)*Hillslope))

The Bottom was constrained to zero.

F.2.2. Results

2H4MBP at 10^{-5} M induced relative luciferase activity of 14.9% and 20.9% in each respective run (Table F-2; Figure F-2). 2H4MBP was considered a "positive" agent as per OPPTS 890.1300 because it exceeded 10% of the response of the positive control.[41] In neither run of the ER transcriptional activation assay did EHMC exceed an increase in luciferase activity greater than 5% (RPC_{max}) at any of the viable concentrations assessed. The EC_{50} (and log thereof) and Hill slopes of the curves using the Hill model constrained at 0 were generated if possible. The top of the curve was constrained to <100% for corticosterone, 17α-methyltestosterone, and the sunscreen ingredients, as they did not exhibit a full response (Table F-2). The reference agents and 2H4MBP-specific luciferase data could be fit to the EC_{50} model. The logEC_{50} for 2H4MBP was −3.2 and −4.0 M, respectively, for runs 1 and 2. The logPC_{10} for 2H4MBP was calculated to be −5.6 M for each respective individual run.

The weak positive control 17α-methyltestosterone exhibited logEC_{50}s of −8.8 and −8.6 M and Hill slopes of >1. 17β-estradiol displayed logEC_{50}s of −10.5 and −10.3 M and Hill slopes of ~1.2 to 1.5. 17α-estradiol displayed logEC_{50}s of −8.8 and −8.6 M and Hill slopes of 1.5 to 2.0 (Table F-2).

F.3. Uterotrophic Assay (OPPTS 890.1600)

F.3.1. Methodology

All procedures were conducted in accordance with OPPTS 890.1600 and were in compliance with the Animal Welfare Act Regulations.[122; 123] Animals were handled and maintained according to the *Guide for the Care and Use of Laboratory Animals*.[124] Briefly, 80 ovariectomized Sprague Dawley (Crl:CD®(SD) IGS) rats, 7 weeks of age, were procured from Charles River Laboratories (Raleigh, NC). Rats were acclimated to the study room for 7 days prior to randomization (body weight stratification to an N of 8/group), and initiation of dosing. Dose formulations of 2H4MBP, EHMC (320 or 1,000 mg/kg/day) and reference agent 17α-ethinyl estradiol (EE) (0.1 mg/kg/day) were prepared in corn oil. Concentrations and homogeneity were confirmed; mean concentrations were within 10% of the target concentration and the homogeneity coefficient of variation was ≤5% of the target concentration.

Rats were administered a dose volume of 5 mL/kg via oral gavage for 3 consecutive days and then humanely euthanized by carbon dioxide (followed by secondary method to confirm death). Body weights and clinical observations were performed at least once daily. At termination, uteri (with cervix) were excised. Uterine wet and blotted weights were recorded to the nearest 0.0001 g. Terminal body weight and body weight gain were analyzed by ANOVA. Uterine and blotted uterine weights were analyzed using a General Linearized Model (JMP, version 12.0.1) with terminal body weight and exposure concentration as model effects. If the probability of the p value was ≤0.05, then potential differences in least square means were identified by Dunnett's test (compared to control). EE was compared to the vehicle using the Student's t-test.

F.3.2. Results

Rats administered 0.1 mg/kg EE or 1,000 mg/kg 2H4MBP displayed statistically significant body weight loss, as well as significant decreases in body weight gain, over the 3-day dosing interval. Terminal body weights of rats exposed to EE were significantly lower than control by 6.4% (Table F-3). Administration of 0.1 mg/kg EE also resulted in statistically significant (2.8- and 2.3-fold) increases in uterine wet and blotted weights, respectively, demonstrating appropriate responsiveness of the system. Neither exposure concentrations of 2H4MBP nor EHMC significantly altered uterine wet or blotted weights.

F.4. Competitive Androgen Receptor Binding (Rat Prostate Cytosol)

F.4.1. Methodology

DMSO is one of the recommended solvents (OPPTS 890.1150) and was selected as a suitable vehicle.[125] 2H4MBP and EHMC solutions were prepared with concentrations up to 10^{-4} M (the limit concentration for the assay) while limiting the final concentration of DMSO in the assay media to ~3.2% (v/v). DMSO was kept at the same concentration for the controls and for the test substance and was tested as a vehicle control with the reference chemical and reference controls for the run as well. Serial dilutions were prepared in DMSO to yield the final assay concentrations of 1×10^{-3} to 1×10^{-10} M. Precipitation of 2H4MBP and EHMC was observed at final assay concentrations of 1×10^{-3} M. The positive control, R1881, strongly binds androgen receptors (ARs) and was included to ensure that the run was properly performed and to allow an assessment of variability in the conduct of the assay across time. R1881 stock (10 mM) was diluted 1:10 and then serial diluted. Final concentrations of unlabeled R1881 were 1×10^{-6} to 1×10^{-11} M. The weak positive control was dexamethasone prepared from a 30 mM stock solution in DMSO and then serially diluted to final concentrations of 1×10^{-3} to 1×10^{-10} M. DMSO concentrations were kept at 3.2%. Visual observation determined the limit of test chemical solubility, and initial compound solubility was determined in solvent. In addition, the solutions were watched closely when added to the experiment tube, as the test compound may precipitate upon addition to the assay tube mixtures.

Cytosol was collected, processed, and validated per EPA OPPTS 890.1150 for use in this study.[125] Briefly, for the three independent runs, ventral prostates from SD rats (90 days old and castrated <1 day prior to collection) were removed, weighed, and sent via overnight carrier on dry ice. Upon receipt, prostates were thawed, inspected for fibrous, inflamed, edematous, or other abnormal appearance, and, if present, the organ was discarded. Prostates were trimmed of excess fascia, weighed, minced with a scalpel blade and fine scissors, and immediately homogenized with a Polytron PT2100 at 4°C in TEDG buffer. The homogenates were centrifuged at 30,000 g for 30 minutes using a Sorvall RC5B centrifuge, cooled to 4°C. The supernatant from a given run was pooled and protein concentration was determined by the Bradford method (BioRad) typical values are 1–4 mg/mL. Samples were frozen and maintained at −70°C until use. Once thawed on ice and diluted to the predetermined optimal protein concentration, they were immediately used. Triamcinolone acetonide was used to prevent progesterone-specific binding.

[^{3}H]R1881 was prepared on the day of assay. The specific activity was adjusted for decay over time prior to performing dilutions, and siliconized tubes were used for the assay. Saturation AR

binding assays were performed for each independent run of the assay. Increasing concentrations of unlabeled R1881 displaced [^{3}H]R1881 from the receptor in a manner consistent with one-site competitive binding; the ligand depletion was held below 15%, and was acceptable as per EDSP OPPTS 890.1150.[125] DMSO at the concentrations used did not alter assay sensitivity or reliability. Resulting data were normalized to % specific binding and plotted. Data were curve fit by weighted least squares nonlinear regression analysis with weights equal to 1/Y according to:

$$Y = Bottom + (Top-Bottom)/(1 + 10\text{^}((\log IC_{50}-X)*Hillslope + \log((Top-Bottom)/(50-Bottom) - 1))).$$

Relative binding activity (RBA) was calculated by dividing the IC_{50} of the positive control R-1881 by the IC_{50} of the respective sunscreen ingredient.

F.4.2. Results

The suitable top concentration of 2H4MBP and EHMC for use in all three independent AR competitive binding assays was 10^{-4} M, as precipitation was seen with both at 10^{-3} M. In the presence of 10^{-4} M 2H4MBP, [^{3}H]-R1881 specific binding was 64.9%, 62.6%, and 61.2% for each independent experiment, with a mean of 62.9%. When fitted to the 1/Y weighted Hill model, the $\log IC_{50}$ ranged from −3.7 to −1.2 M. Collectively, 2H4MBP tested up to 10^{-4} M did not displace more than 50% of the [^{3}H]-R1881, categorizing 2H4MBP as "equivocal" (Table F-4; Figure F-3), as per OPPTS Guidance.[125]

In each of the three AR competitive binding assays and the averaged competition curve, the [^{3}H]-R1881 specific binding at every soluble EHMC concentration tested was >75%, with $\log IC_{50}$ values ranging from −2.2 to 5.0 M. Therefore, EHMC was classified as a "nonbinder." The weak positive control dexamethasone exhibited $\log IC_{50}$ values of −4.3 to −4.6 M, whereas R1881-1 displayed $\log IC_{50S}$ of −8.9 to −9.9 M. The Hill slopes for these reference agents were approximately −1. The mean RBA for the "equivocal" 2H4MBP UV filter was >0.0002%. The mean RBA for the weak reference agent dexamethasone was 0.0024%.

F.5. Androgenic Transactivation Activity in MDA-kb2 Reporter Cells: Agonist and Antagonist Modes

F.5.1. Methodology

DMSO was selected as a suitable vehicle for the test substances. Therefore, solutions with a test substance concentration of up to 10^{-3} M (the highest concentration assessed) can be prepared while limiting the final concentration of DMSO in the assay medium to 0.5% (v/v). Each test substance was prepared for addition to the cell system by making a 400 mM stock. Dilutions were prepared in DMSO up to 400x, the final target concentration. Dihydrotestosterone (DHT), nilutamide (Nil), dichlorodiphenyldichloroethylene (p,p'-DDE, or DDE), 2H4MBP, and EHMC were all prepared on the day of dosing. Considering the short-term nature of studies of this type, no analyses of stability, homogeneity, or achieved concentration(s) were carried out on preparations of the test substance or positive control chemicals, either before or after the treatment phase. Solubility limits were determined by visual observation or particulate light scattering via nephelometry with Nepheloskan. Stably transfected MDA-kb2 cell line was obtained from ATCC and confirmed to be mycoplasma-free. The MDA-kb2 cell line is derived

from the MDA-MB-453 breast cancer cell line by stable transfection with a mouse mammary tumor virus luciferase-neo reporter construct containing the androgen response element. The transcriptional stability was monitored by the use of the following reference chemicals: DHT, Nil, and p,p'-DDE. A complete concentration response curve for each reference compound was run each time the transcriptional activation assay was performed. Cells were maintained in Leibovitz's L-15 culture medium containing 10% fetal bovine serum, in an incubator at approximately 37°C. The MDA-kb2 cell line is not contact inhibited and can be grown to confluence. Cells were subcultivated at a 1:2 to 1:8 subcultivation ratio. The cells were suspended with complete medium and plated into wells of a 96-well cell culture plate at a density of approximately 1×10^4 cells/100 μL/well. The cells were then placed into an incubator at approximately 37°C overnight prior to chemical exposure.

After adding the reference chemicals/test substances, the plates were incubated in an incubator at approximately 37°C for approximately 24 hours. For the agonism plates, all concentrations were tested in replicates of 6/plate. In addition, for each concentration, two replicates/plate were prepared that incorporated the AR antagonist Nil. Replicates incorporating an AR antagonist allow for the identification of nonspecific (i.e., non-AR-mediated) induction of the luciferase gene as true AR-mediated induction is inhibited by addition of an antagonist whereas nonspecific induction is not. For the antagonism plates, all concentrations were tested in replicates of 4/plate. Four replicates were co-administered 1 nM DHT and test article at each concentration. Four replicates were co-administered 1,000 nM DHT and test article at each concentration. Replicates incorporating 1,000 nM DHT allowed for the identification of assay interference. Cell viability was monitored by a two-read propidium iodide (PI) uptake assay conducted under low light conditions. Cells were seeded and exposed as described above in a black-walled 96-well cell culture plate.

Digitonin (125 μM) was used as a cell death positive control. Following chemical exposure, the growth medium was removed and 50 μL of a PI working solution (44 μM in phosphate buffered saline) was added to each well. Background fluorescence was evaluated by measuring fluorescence immediately on a Packard Fusion fluorescence plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Following this determination, 50 μL of a 2% (v/v) Triton X-100 solution was added to each well and the plate was incubated at room temperature for approximately 15 minutes to fully lyse all cells in the wells before measuring fluorescence at the same wavelengths. The background-corrected fluorescence was calculated for each well by subtracting the results of the first read from the results of the second read. The change in cell viability was determined by comparing treated wells to the vehicle control wells. A ≥20% reduction in cell viability was considered evidence of cytotoxicity.

Luciferase activity was determined as described by Wilson[116]. Acceptance criteria included: (1) background value ratio of vehicle control to antagonist control should be <10X, and (2) the ratio of positive control to vehicle control should be >3X. Each data point was normalized to the average of the vehicle-only treated control (fold induction). Where appropriate, $\log PC_{50}$, $\log PC_{10}$, $\log EC_{50}$, and Hill slope values were calculated. For the test substance, the maximum response relative to the positive control (RPC_{max}) was determined. In each individual run of the transcriptional activation assay, if RPC_{max} was less than 20%, the test substance was considered a negative response for AR agonism. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria: (1) the mean

normalized luciferase signal of the PC (10 nM DHT) should be at least fourfold that of the mean vehicle control on each plate, and (2) the results of the reference compounds, Nil and DHT, should be within the acceptable ranges. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered definitive. The test substance was considered negative if RPC_{max} was <20% in at least two definitive runs of the transcriptional activation assay. The test substance was considered positive if RPC_{max} was ≥20% in at least two definitive runs of the transcriptional activation assay.

To determine the relative transcriptional activity as compared to the PC (10 nM DHT), the luminescence data from each plate were analyzed as follows: wells incorporating 1 nM DHT were analyzed in an identical fashion to wells incorporating 1,000 nM DHT, except that the data were normalized to the induced control with 1 nM DHT or 1,000 nM DHT, respectively. Any cytotoxic concentrations were excluded from data analysis. The mean value for the vehicle control wells was calculated and subtracted from each well to normalize the data. The mean value for the induced control with 1 nM or 1,000 nM DHT was calculated. The wells dosed with test or control substance and 1 nM or 1,000 nM DHT were normalized to the mean value for the induced control with 1 nM or 1,000 nM DHT, respectively. Averages of antagonist or high agonist control % maximal induction were calculated (test or control substance with 1 nM or 1,000 nM DHT, respectively). Differentials were calculated (averages of high agonist % maximal induction control minus averages of antagonist % maximal induction control). Where appropriate, RIC_{max}, IC_{50}, and Hill slope values were calculated. If the differential between the high antagonism and the low antagonism was greater than 50% and had a dose response (more than one data point) in two of two runs, then the test substance was considered positive. If the differential between the high antagonism and the low antagonism was less than 50% and did not have a dose response (more than one data point) in two of two runs, than the test substance was considered negative. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria: the mean normalized luciferase signal of the PC (10 nM DHT) should have been at least fourfold that of the negative control on each plate. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered to be definitive.

F.5.2. Results

In all independent runs of the agonist transcriptional activation assay, neither 2H4MBP nor EHMC resulted in an increase in luciferase activity at any of the viable soluble concentrations tested; RPC_{max} <20% (Figure F-4).

In two of three runs the decrease in DHT-induced luciferase activity resulting from 2H4MBP exposure was approximately 25% at the highest feasible dose of −4.5 M, with the first run exhibiting a luciferase activity of 72.2% of maximal (Figure F-5). EHMC had no apparent inhibitory effect on DHT-induced AR transcriptional activity. The EC_{50}, IC_{50} (and logs thereof), and Hill slopes of the curves using the unconstrained Hill model were determined (Table F-5). Curves could be fit for two of the three 2H4MBP runs and both EHMC runs. The $logIC_{50}$s and Hill slopes for Nil and DDE were approximately −6.4 and −4.8 M, −1.3 and −1, respectively.

F.6. Hershberger Bioassay

F.6.1. Methodology

All procedures were conducted in accordance with OPPTS 890.1400 and in compliance with the Animal Welfare Act Regulations.[122; 126] Animals were handled and maintained according to the *Guide for the Care and Use of Laboratory Animals*.[124] Briefly, 208 (total) Sprague Dawley Crl:CD(SD) IGS rats were procured from Charles River Laboratories (Raleigh, NC) one week after PND 45 castration surgery. Rats were acclimated to the study room for 7 days prior to randomization (body weight stratification; N = 8/group) and initiation of dosing. This assessment was done as two separate studies (with respective controls). For the androgen agonist assessment, 0.4 mg/kg/day of testosterone propionate (TP) was used as the positive inducer of maximal androgenic response, whereas 3 mg/kg/day of flutamide (FT) (in combination with 0.4 mg/kg/day TP) was used to induce maximal inhibition of the TP-induced androgenic response. Dose formulations of 2H4MBP, EHMC, TP, and flutamide were prepared in corn oil.

In the first study, the vehicle (corn oil) and two exposure concentrations of 2H4MBP and EHMC were used (320 and 1,000 mg/kg/day) for the assessment androgen agonism, and three exposure concentrations (100, 320, and 1,000 mg/kg/day) were used to assess potential sunscreen ingredient antagonism of TP-induced androgen action. Respective controls (vehicle, TP, TP and FT) were included in each study. OPPTS 890.1400 specifies to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after 10 consecutive days of chemical administration, and the highest dose should not cause a reduction in the final body weight of the animals greater than 10% of control weight.[126] 2H4MBP and EHMC were evaluated up to the limit exposure concentration (1,000 mg/kg/day). The test substance, FT, or corn oil dose formulations were administered by oral gavage at a dose volume of 5 mL/kg body weight. TP dose formulations were administered by subcutaneous injection into the dorsoscapular region at a dose volume of 0.5 mL/kg body weight. In co-administered animals, oral gavage preceded subcutaneous injections. Dosing occurred 24 hours (± 2 hours) from the previous dose. Dose volume was determined on individual animal daily body weight. The dosing sequence was stratified across dose groups—one animal from each group and then repeated until all animals were dosed. Selection of the route of administration was in accordance with OPPTS 890.1400 and OECD Guideline 441.[127; 126]

Samples were collected for confirmation of concentration and homogeneity. Mean concentrations were within 10% of the target concentration, and the homogeneity coefficient of variation was less than ≤5% of the target concentration. Rats were orally gavaged at a dose volume of 5 mL/kg for 10 consecutive days. Body weights and clinical observations were performed at least once daily. At study termination, rats were humanely euthanized by CO_2 asphyxiation and death confirmed by a second method in the same order as they were dosed.

Gross observations were recorded for the ventral prostate, seminal vesicle and coagulating gland with fluid, levator ani/bulbocavernosus muscle, Cowper's gland, and glans penis. The tissues were excised, trimmed of excess adhering tissue and fat, and weighed to the nearest 0.0001 g.

Terminal body weight and body weight gain were analyzed by ANOVA. Organ weights were tested (by dose) and assumed to be normally distributed and analyzed by General Linearized Model (JMP version 12.0.1) with terminal body weight and exposure concentration as model

effects. If the probability of the p value (versus the respective control) was ≤0.05 then potential differences in least square means were identified by Dunnett's test. TP was compared to vehicle using the Student's t-test.

F.6.2. Results

In the absence of androgenic action, neither 2H4MBP nor EHMC had any effect on androgen dependent organ weights, demonstrating that 2H4MBP and EHMC do not exhibit any in vivo androgenic activity (Table F-6; Table F-7). In the presence of TP, EHMC did not attenuate the expected androgen-mediated increase in organ weights, demonstrating that EHMC does not exhibit any antiandrogenic activity in vivo under the exposure concentrations assessed. Rats co-administered 1,000 mg/kg/day of 2H4MBP and TP displayed statistically lower day 10 body weight and body weight gain (7 and 28%, respectively) relative to control. The mean weights of the glans penis and ventral prostate were also statistically lower (6 and 17%, respectively). The weight of the seminal vesicles was also slightly lower. However, when concurrent body weight is used as a covariate, the magnitude of the response is decreased and no longer attains statistical significance. Given that these organ weight changes only occurred in the presence of lower body weights at the highest dose assessed, these organ weight changes are likely secondary to effects on body weight.

F.7. Human Recombinant Aromatase Assay

F.7.1. Methodology

The potential for 2H4MBP and EHMC to as act as inhibitors of aromatase activity was assessed using human CYP19 (aromatase) and P450 reductase Supersomes™ (Gentest™). The substrate for the assay was [1β-^{3}H]-Androstenedione (ASDN), which is then converted by aromatase to estrone. Estrone was extracted via aqueous phase and quantified utilizing liquid scintillation counting, Final concentrations of EHMC and 2H4MBP in the aromatase assay were 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, $10^{-4.5}$, 10^{-4}, and 10^{-3} M. Three independent runs of the aromatase assay were conducted. In each independent run, each concentration of test substance was tested in triplicate. In addition, the positive control inhibitor 4-hydroxyandrostenedione (4OH-ASDN) was included each time the aromatase assay was performed. Increasing concentrations of 4OH-ASDN decrease the aromatase activity in a concentration dependent manner.

F.7.2. Results

According to the data interpretation procedure outlined by the EPA (OPPTS 890.1200), EHMC was classified as a noninhibitor, with mean aromatase activity of 100% (±6% SD).[128] 2H4MBP was classified as equivocal, as it produced a mean aromatase activity level of 51% (±13% SD) of control activity at the highest soluble test concentration of 10^{-4} M.

F.8. H295R Steroidogenesis Assay

F.8.1. Methodology

The ability of 2H4MBP or EHMC to affect the steroidogenic pathway, beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone and estradiol/estrone was assessed using the H295R human adrenocarcinoma cell

line. The final concentrations of each compound tested in the steroidogenesis assay were: 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM. Four independent runs of the steroidogenesis assay were conducted. Three of the four assays were analyzed for each compound. All test chemicals, reference chemicals, and solvent controls were tested in replicates of 3/plate, with the exception of the solvent controls on the quality control (QC) plate. Six solvent control wells were analyzed on the QC plate. The H295R supplemented medium used in the assay at the time of plating, dosing, and harvest contained 10 μM 22R-hydroxycholesterol. The duration of exposure was 48 hours. A QC plate containing two doses of reference chemicals forskolin and prochloraz was run each time the assay was performed. Cell viability was assessed after the 48-hour exposure using the MTT assay. Testosterone and estradiol concentrations were measured using HPLC/MS-MS by OpAns, LLC (Durham, NC). All concentrations that exhibited greater than 20% cytotoxicity in the MTT cell viability assay were excluded from the statistical analysis of testosterone and estradiol concentrations.

F.8.2. Results

The highest concentration of 2H4MBP that could be tested in the assays was 100 μM, according to apparent solubility and cytotoxicity results. Statistically significant induction of testosterone was observed at the 100 μM concentration in all three runs of the assay. Statistically significant estradiol induction was observed at 10 and 100 μM in three runs, and at 0.1, 1, 10, and 100 μM in one run. Although statistically significant results were identified at the 100 μM 2H4MBP concentration in all three runs, precipitation was noted under the microscope after the 48-hour exposure period at this concentration. Nonetheless, 2H4MBP would be classified as positive in the steroidogenesis assay for effects on estradiol on the basis of the data interpretation criteria outlined in the OECD test guideline for the assay. Induction of testosterone production was apparently observed at 100 μM, concomitant with precipitation 2H4MBP. The criteria outlined in the OECD test indicate that the effects of 2H4MBP on testosterone in each run would be classified as equivocal.

The highest concentration of EHMC that could be tested in the assays was 0.1 μM in run 1 and 100 μM in all runs 2 and 3, as per solubility results. Precipitation was observed prior to exposure in run 1 at the 1, 10, and 100 μM concentrations. Cytotoxicity greater than 20% was not observed in any of the three runs at any of the concentrations tested. No statistically significant effects were observed on testosterone or estradiol production at any of the concentrations that were analyzed in any of the three runs. According to the data interpretation criteria outlined in the OECD test guideline, EHMC is negative in the steroidogenesis assay.

Table F-1. Uterine Estrogen Receptor Binding Assay Parameters and Curve Fit[a]

	Reference Agents			UV Filter	
	17β-Estradiol	**19-Norethindrone**	**Octyltriethoxysilane**	**2H4MBP**	**EHMC**
RPC_{max}					
Exp. 1	0.0 ± 0.4	0.9 ± 0.1	94.1 ± 1.8	83.2 ± 0.9	89.2 ± 2.9
Exp. 2	0.0 ± 0.2	0.8 ± 0.1	88.6 ± 1.4	76.5 ± 0.6	93.4 ± 0.8
Exp. 3	0.0 ± 0.1	1.6 ± 0.6	90.9 ± 1.6	77.2 ± 0.4	93.1 ± 2.1
Mean	0.0 ± 0.0	1.1 ± 0.3	91.2 ± 1.6	79.0 ± 2.1	91.9 ± 1.4
PC_{max} (M)					
Exp. 1	10^{-7}	10^{-4}	$10^{-6.5}$	10^{-4}	10^{-4}
Exp. 2	10^{-7}	10^{-4}	$10^{-4.5}$	10^{-4}	10^{-4}
Exp. 3	10^{-7}	10^{-4}	$10^{-4.5}$	10^{-4}	10^{-4}
R^2					
Exp. 1	0.9987	0.9993	Ambiguous 0.2727	Ambiguous 0.9915	Ambiguous 0.8190
Exp. 2	0.9973	0.9981	Ambiguous 0.1109	0.8872	Ambiguous 0.5173
Exp. 3	0.9953	0.9998	Ambiguous 0.3306	Ambiguous 0.9843	Ambiguous 0.3545
Top					
Exp. 1	94.7 ± 1.9	93.1 ± 0.9	~7,305	95.3 ± 0.3	97.8 ± 2.2
Exp. 2	92.5 ± 2.8	96.6 ± 2.1	91.8 ± 2.2	97.2 ± 1.8	844,702
Exp. 3	95.7 ± 4.0	100.3 ± 0.5	98.9 ± 2.7	98.6 ± 0.6	98.7 ± 1.4
Bottom					
Exp. 1	−0.5 ± 2.0	−0.7 ± 1.5	~40	~49	~50
Exp. 2	−1.1 ± 2.9	−7.8 ± 4.5	~50	<0	~41
Exp. 3	−5.0 ± 5.0	−1.0 ± 0.8	~50	~49	~50
$LogIC_{50}$					
Exp. 1	−9.0 ± 0.0	−5.5 ± 0.0	~296.6	~−3.6	~4.7
Exp. 2	−9.0 ± 0.0	−5.5 ± 0.0	~−2.6	−2.9 ± 4.0	~−116.6
Exp. 3	−8.8 ± 0.1	−5.6 ± 0.0	~−2.7	~−2.8	~−2.7

	Reference Agents			UV Filter	
	17β-Estradiol	**19-Norethindrone**	**Octyltriethoxysilane**	**2H4MBP**	**EHMC**
IC_{50}					
Exp. 1	9.9×10^{-10}	2.7×10^{-6}	3.7×10^{297}	$\sim 2.3 \times 10^{-4}$	4.6×10^{4}
Exp. 2	8.8×10^{-10}	2.2×10^{-6}	$\sim 2.5 \times 10^{-3}$	11.4×10^{-4}	~0
Exp. 3	1.3×10^{-9}	2.3×10^{-6}	$\sim 1.9 \times 10^{-3}$	$\sim 14.8 \times 10^{-4}$	$\sim 1.9 \times 10^{-3}$
Hill Slope					
Exp. 1	−1.1 ± 0.1	−1.1 ± 0.1	~0	~−5.6	−0.4 ± 1.0
Exp. 2	−1.0 ± 0.1	−0.7 ± 0.1	~−4.4	−0.5 ± 0.9	~0
Exp. 3	−0.8 ± 0.1	−0.9 ± 0.0	~−4.5	~−1.6	~−4.7
Log Relative Binding Affinity					
Exp. 1	–	1.6	NS	2.5	−1.9
Exp. 2	–	1.6	3.5	3.1	0.1
Exp. 3	–	1.6	3.3	3.2	3.3
Relative Binding Affinity (%)					
Exp. 1	–	0.0385	–	0.0004	0.0000
Exp. 2	–	0.0409	0.0001	0.0001	–
Exp. 3	–	0.0565	0.0002	0.0001	0.0001

2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate.
RPC_{max} = maximum level of response induced by test chemical compared to positive control; PC_{max} = maximum response of test chemical relative to response induced by 1 nM 17β-Estradiol.
[a]Values reported as average ± SEM.
Curve Fit: Y = Bottom + (Top−Bottom)/(1 + 10^((logIC_{50}−X)*Hillslope + log((Top−Bottom)/(50−Bottom) −1))); Bottom > 0 for sunscreen ingredients.

Table F-2. Estrogen Receptor Transcriptional Activation, Assay Parameters, and Curve Fit[a]

	Reference Agents				UV Filters	
	17β-Estradiol	**17α-Ethynyl Estradiol**	**Corticosterone**	**17α-Methyltestosterone**	**2H4MBP**	**EHMC**
RPC_{max}						
Exp. 1	125.8 ± 9.4	103.8 ± 11.6	2.3 ± 0.5	43.9 ± 8.0	14.9 ± 1.1	4.1 ± 0.8
Exp. 2	135.3 ± 10.4	101.5 ± 7.0	0.0 ± 0.0	35.4 ± 4.0	20.9 ± 3.1	3.2 ± 0.4
PC_{max}						
Exp. 1	10^{-8}	10^{-7}	10^{-7}	10^{-5}	10^{-5}	10^{-8}
Exp. 2	10^{-9}	10^{-8}	10^{-7}	10^{-5}	10^{-5}	10^{-8}
R^2						
Exp. 1	0.9966	0.9962	Not converged	0.9588	0.8897	Interrupted
Exp. 2	0.9930	0.9959	Not converged	0.9602	0.9062	Interrupted
Top						
Exp. 1	120.8 ± 2.9	99.0 ± 2.5	–	–	–	–
Exp. 2	129.3 ± 4.6	115.0 ± 3.0	–	–	–	–
$LogEC_{50}$						
Exp. 1	−10.5 ± 0.1	−8.8 ± 0.1	–	−4.9 ± 0.1	−3.2 ± 0.5	$\sim -6.6 \times 10^{5}$
Exp. 2	−10.3 ± 0.1	−8.6 ± 0.1	–	−4.6 ± 0.1	−4.0 ± 0.3	$\sim -4 \times 10^{5}$
EC_{50}						
Exp. 1	3.3×10^{-11}	1.6×10^{-9}	–	1.4×10^{-5}	6.4×10^{-4}	~0
Exp. 2	4.6×10^{-11}	2.6×10^{-9}	–	2.3×10^{-5}	1.0×10^{-4}	~0
Hill Slope						
Exp. 1	1.5 ± 0.2	2.0 ± 0.8	–	0.9 ± 0.2	0.4 ± 0.1	~0
Exp. 2	1.2 ± 0.2	1.5 ± 0.2	–	0.7 ± 0.1	0.6 ± 0.2	~0
$LogPC_{10}$						
Exp. 1	−11.5	−9.7	NC	−6.0	−5.6	NC
Exp. 2	−11.6	−9.7	NC	−6.0	−5.6	NC
PC_{10}						
Exp. 1	7.6×10^{-12}	4.8×10^{-10}	NC	1.1×10^{-6}	2.6×10^{-6}	NC
Exp. 2	6.6×10^{-12}	6.4×10^{-10}	NC	1.1×10^{-6}	2.6×10^{-6}	NC

2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate; NC = Not calculated.
[a]Values reported as average ± SEM.
Curve Fit: Y = Bottom + (Top−Bottom)/(1 + 10^((logEC$_{50}$−X)*Hillslope)). Constrained at 0 (17β-estradiol, 17α-ethynyl estradiol) or 0 and 100 (corticosterone, 17α-methyltestosterone, 2H4MBP, EHMC).

Table F-3. Uterine and Body Weights of Ovariectomized Sprague Dawley Rats Administered UV Sunscreen Ingredients via Gavage for Three Days[a]

	0 mg/kg	EE 0.1 mg/kg[b]	2H4MBP 320 mg/kg	2H4MBP 1,000 mg/kg	EHMC 320 mg/kg	EHMC 1,000 mg/kg
n	8	8	8	8	8	8
Uterine Weight (mg)[c]	94.3 ± 14.5	277.1 ± 76.3*	94.3 ± 11.2	103.8 ± 19.0	89.1 ± 11.2	94.4 ± 9.5
Uterine Weight, Blotted (mg)[c]	86.8 ± 13.7	210.1 ± 27.5*	87.5 ± 10.5	96.3 ± 17.9	82.2 ± 11.5	87.5 ± 9.7
Body Weight (Day 1) (g)	257.7 ± 12.9	260.9 ± 12.8	257.8 ± 13.4	256.2 ± 11.6	260.7 ± 10.6	259.5 ± 14.5
Terminal Body Weight (g)[d]	268.1 ± 10.2	251.4 ± 12.7*	268.8 ± 13.1	254.6 ± 9.4	271.5 ± 9.0	264.8 ± 12.0
Body Weight Gain (g)[d]	10.4 ± 6.6	−7.0 ± 5.7*	11.1 ± 2.9	−1.6 ± 5.8*	10.8 ± 6.3	5.3 ± 6.3

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control.
*Statistically significantly at $p \leq 0.05$.
2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate.
Initial mean body weights were ~260 g.
[a]Data presented as mean ± SD.
[b]EE was compared to control using the Student's t-test.
[c]Uterine and blotted uterine weights were analyzed by MS Excel and SAS version 9.1 (SAS Institute, Cary, NC) with terminal body weight and exposure concentration as model effects. If the probability of the p value was <0.05 then potential differences in least square means were identified by Dunnett's test (compared to control).
[d]Terminal body weight and body weight gain were analyzed by ANOVA.

Table F-4. Prostate Androgen Receptor Binding Assay Parameters and Curve Fit[a]

	Reference Agents		UV Filter	
	R1881-1	**Dexamethasone**	**2H4MBP**	**EHMC**
Specific Binding Response$_{max}$				
Exp. 1	1.2 ± 1.0	2.9 ± 1.6	64.9 ± 1.4	84.5 ± 2.1
Exp. 2	1.9 ± 1.0	5.1 ± 1.2	62.5 ± 0.4	81.9 ± 2.2
Exp. 3	0.0 ± 0.8	0.5 ± 0.7	61.3 ± 4.0	89.3 ± 1.4
Concentration$_{max}$ (M)				
Exp. 1	10^{-6}	10^{-3}	10^{-4}	10^{-4}
Exp. 2	10^{-6}	10^{-3}	10^{-4}	10^{-4}
Exp. 3	10^{-6}	10^{-3}	10^{-4}	10^{-4}
R^2				
Exp. 1	0.9993	0.9990	Ambiguous 0.9927	Ambiguous 0.9769
Exp. 2	0.9993	0.9996	Ambiguous 0.9645	Ambiguous 0.9445
Exp. 3	0.9890	0.9997	Hit constraint 0.9872	0.9360
Top				
Exp. 1	98.7 ± 2.6	98.6 ± 1.7	99.9 ± 1.0	100.1 ± 1.1

	Reference Agents		UV Filter	
	R1881-1	**Dexamethasone**	**2H4MBP**	**EHMC**
Exp. 2	113.3 ± 4.9	98.7 ± 0.9	92.6 ± 1.4	95.8 ± 0.8
Exp. 3	99.0 ± 11.5	94.0 ± 1.0	100.4 ± 1.2	100.0 ± 0.9
Bottom				
Exp. 1	1.2 ± 0.3	−2.9 ± 2.1	~50.0	~50.0
Exp. 2	1.8 ± 0.2	−0.1 ± 1.2	~50.0	~50.0
Exp. 3	−0.3 ± 0.4	−2.0 ± 0.5	~0	~0
LogIC$_{50}$				
Exp. 1	−8.9 ± 0.0	−4.4 ± 0.0	~−1.2	~5.0
Exp. 2	−9.9 ± 0.0	−4.3 ± 0.0	~−3.1	~−0.7
Exp. 3	−9.1 ± 0.1	−4.6 ± 0.0	−3.7 ± 0.5	−2.2 ± 9.5
IC$_{50}$				
Exp. 1	1.3×10^{-9}	3.8×10^{-5}	6.7×10^{-2}	$\sim 9.5 \times 10^{4}$
Exp. 2	1.1×10^{-10}	4.7×10^{-5}	8.1×10^{-4}	~0.2
Exp. 3	0.9×10^{-9}	2.5×10^{-5}	1.8×10^{-4}	$\sim 6.2 \times 10^{-3}$
Hill Slope				
Exp. 1	−0.9 ± 0.1	−0.9 ± 0.1	−1.1 ± 0.2	−0.4 ± 0.1
Exp. 2	−0.9 ± 0.0	−1.0 ± 0.1	~−3.1	~−1.2 ± 1.7
Exp. 3	−0.9 ± 0.2	−1.0 ± 0.1	−0.8 ± 0.8	−0.5 ± 0.3
LogRBA				
Exp. 1	–	2.0	7.4	−1.8
Exp. 2	–	2.3	3.2	14.1
Exp. 3	–	2.0	2.4	4.1
Mean (Exp. 1–3)	–	2.1	4.3	5.5
RBA (%)				
Exp. 1	–	0.0034	<0.0000	<0.0000
Exp. 2	–	0.0002	<0.0000	<0.0000
Exp. 3	–	0.0036	0.0005	<0.0000
Mean (Exp. 1–3)	–	0.0024	0.0002	<0.0000

2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate.
[a]Data presented mean ± SEM.
Curve Fit: Y = Bottom + (Top−Bottom)/(1 + 10^((logIC$_{50}$−X)*Hillslope + log((Top−Bottom)/(50−Bottom) −1))). 1/Y weighed; constrained at Bottom >0 for 2H4MBP and EHMC.

Table F-5. Androgen Receptor Transactivation: Antagonist Mode Parameters and Curve Fit

	Reference Agents		UV Filter	
	DDE	**Nilutamide**	**2H4MBP**	**EHMC**
RPC_{min}				
Exp. 1	10.3 ± 0.3	−1.9 ± 0.0	72.2 ± 4.6	98.5 ± 7.9
Exp. 2	13.2 ± 1.0	−2.1 ± 0.0	89.0 ± 2.8	83.3 ± 5.2
Exp. 3	13.9 ± 1.0	−1.0 ± 0.4	75.6 ± 5.0	–
PC_{max}				
Exp. 1	10^{-4}	10^{-4}	$10^{-4.5}$	$10^{-4.5}$
Exp. 2	10^{-4}	10^{-4}	$10^{-4.5}$	$10^{-4.5}$
Exp. 3	10^{-4}	10^{-4}	$10^{-4.5}$	–
R^2				
Exp. 1	0.9992	0.9979	Ambiguous 0.7991	Ambiguous 0.3451
Exp. 2	0.9795	0.9960	Not converged	Ambiguous 0.3581
Exp. 3	0.9808	0.9879	Ambiguous 0.5396	–
Top				
Exp. 1	111.8 ± 1.1	97.8 ± 2.6	108.6 ± 11.6	114.4 ± 22.0
Exp. 2	94.6 ± 3.2	101.5 ± 3.7	–	94.3 ± 4.4
Exp. 3	110.8 ± 4.1	116.1 ± 15.3	110.6 ± 7.7	–
Bottom				
Exp. 1	8.4 ± 2.3	2.0 ± 1.4	~50.0	~50.0
Exp. 2	14.7 ± 7.4	1.6 ± 2.0	–	~50.0
Exp. 3	11.2 ± 10.7	2.3 ± 3.8	~50.0	–
$LogIC_{50}$				
Exp. 1	−4.9 ± 0.0	−6.4 ± 0.0	~−3.2	~−1.2
Exp. 2	−4.9 ± 0.1	−6.3 ± 0.0	–	~−1.9
Exp. 3	−4.8 ± 0.1	−6.5 ± 0.1	~−4.3	–
IC_{50}				
Exp. 1	1.4×10^{-5}	4.3×10^{-7}	$\sim 0.7 \times 10^{-4}$	$\sim 6.7 \times 10^{-2}$
Exp. 2	1.3×10^{-5}	4.6×10^{-7}	–	$\sim 1.2 \times 10^{-2}$
Exp. 3	1.5×10^{-5}	3.1×10^{-7}	$\sim 5.1 \times 10^{-5}$	–
Hill Slope				
Exp. 1	−1.6 ± 0.1	−1.6 ± 0.2	−2.6 ± 3.9	−1.2 ± 4.8
Exp. 2	−2.1 ± 0.8	−1.7 ± 0.2	–	−1.6 ± 3.4
Exp. 3	−1.5 ± 0.5	−1.0 ± 0.3	~−16.7	–

DDE = dichlorodiphenyldichloroethylene; 2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate.
Curve Fit: Y = Bottom + (Top−Bottom)/(1 + 10^((logIC$_{50}$−X)*Hillslope + log((Top−Bottom)/(50−Bottom) −1))); unconstrained.

Table F-6. Reproductive Organ and Body Weights of Rats Administered 2H4MBP or EHMC via Gavage for 10 Days (Agonist Assessment)[a]

	0 mg/kg	TP 0.4 mg/kg	2H4MBP 320 mg/kg	2H4MBP 1,000 mg/kg	EHMC 320 mg/kg	EHMC 1,000 mg/kg
n	7[b]	8	8	8	8	8
Organ Weight (mg)						
Glans Penis[c]	47.4 ± 6.1	92.7 ± 5.0*	52.4 ± 7.3	50.7 ± 5.2	49.4 ± 7.0	50.4 ± 5.9
Cowper's Gland[d]	5.4 ± 1.5	39.0 ± 5.6*	5.2 ± 2.6	5.1 ± 1.6	4.6 ± 1.5	5.6 ± 2.0
LABC[d]	133.4 ± 34.9	384.5 ± 51.7*	133.4 ± 19.1	123.4 ± 32.0	143.3 ± 28.9	129.1 ± 25.6
Ventral Prostate[c]	15.8 ± 3.1	186.2 ± 57.2*	15.2 ± 3.0	14.0 ± 1.6	16.1 ± 2.4	15.6 ± 2.8
Seminal Vesicles[c]	31.1 ± 5.3	542.5 ± 63.2*	39.2 ± 9.0	33.6 ± 9.6	34.1 ± 6.4	33.9 ± 7.6
Body Weight (g)						
Day 1	255.5 ± 14.3[e]	259.3 ± 11.9	255.9 ± 17.7	258.7 ± 12.4	256.8 ± 14.4	257.4 ± 9.3
Day 11	302.2 ± 24.9	335.1 ± 24.1*	299.0 ± 27.7	298.0 ± 20.3	313.6 ± 29.4	298.3 ± 14.0
Weight Gain	47.3 ± 16.6	75.8 ± 16.7*	43.1 ± 10.6	39.4 ± 11.2	56.8 ± 17.6	40.9 ± 10.4

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p < 0.05$ when compared to the respective vehicle control group.
^Statistically significant at $p < 0.05$ when compared to the respective TP control.
2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate; LABC = levator ani/bulbocavernosus muscle; TP = testosterone propionate.
[a]Data presented as mean ± SD.
[b]Decrease in N due to animal removal.
[c]Statistical analysis performed by ANOVA. If significant, the means were subjected to Dunnett's test (compared to vehicle control) using JMP version 12.0.1.
[d]Statistical analysis performed using Kruskal Wallace nonparametric analyses. If significant, the means were subjected to Dunn's test (compared to vehicle control).
[e]Average weight of all eight animals in the 0 mg/kg group at study initiation.

Table F-7. Reproductive Organ and Body Weights of Rats Administered 2H4MBP or EHMC via Gavage for 10 Days (Antagonist Assessment)[a]

	0 mg/kg (+ TP)b	FT 3 mg/kg (+ TP)	2H4MBP 100 mg/kg (+ TP)	2H4MBP 320 mg/kg (+ TP)	2H4MBP 1,000 mg/kg (+ TP)	EHMC 100 mg/kg (+ TP)	EHMC 320 mg/kg (+ TP)	EHMC 1,000 mg/kg (+ TP)
n	8	8	8	8	8	8	6[c]	8
Organ Weight (mg)								
Glans Penis[d]	92.7 ± 5.0*	62.5 ± 3.8^[d]	95.6 ± 4.7	94.0 ± 4.5	87.5 ± 3.8^	94.7 ± 7.8	94.6 ± 3.2	91.6 ± 5.0
Cowper's Gland[e]	39.0 ± 5.6*	12.2 ± 3.9^	38.1 ± 5.2	37.2 ± 5.6	37.3 ± 9.8	39.4 ± 5.7	36.7 ± 7.7	32.1 ± 5.0
LABC[e]	384.5 ± 51.7*	181.7 ± 25.6^	419.5 ± 41.9	428.0 ± 55.1	387.9 ± 41.1	415.9 ± 46.3	419.1 ± 42.6	359.4 ± 47.3
Ventral Prostate[d]	186.2 ± 57.2*	37.0 ± 7.7^	206.2 ± 38.6	195.8 ± 32.1	155.0 ± 21.3^	182.4 ± 47.6	176.9 ± 47.2	147.6 ± 39.7
Seminal Vesicles[d]	542.5 ± 63.2*	58.5 ± 16.7^	594.7 ± 54.5	618.8 ± 97.3	506.5 ± 39.9	562.3 ± 74.4	610.1 ± 99.1	465.5 ± 80.2
Body Weight (g)								
Day 1	259.3 ± 11.9	258.1 ± 14.4	257.9 ± 14.2	260.8 ± 9.5	256.8 ± 13.4	259.9 ± 14.7	259.4 ± 11.1	259.3 ± 13.3
Day 11	335.1 ± 24.1*	324.0 ± 24.3	338.1 ± 17.7	339.2 ± 15.9	311.2 ± 16.7^	330.2 ± 42.3	343.7 ± 26.5	319.7 ± 24.8
Weight Gain	75.8 ± 16.7*	65.9 ± 14.2	80.2 ± 9.0	78.4 ± 10.1	54.4 ± 12.4^	70.3 ± 30.9	85.2 ± 23.9	60.4 ± 16.4

Statistical significance for a treatment group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p < 0.05$ when compared to the respective vehicle control group.
^Statistically significant at $p < 0.05$ when compared to the respective TP control.
2H4MBP = 2,4-dihydroxy-4-methoxybenzophenone; EHMC = 2-ethylhexyl p-methoxycinnamate; LABC = levator ani/bulbocavernosus muscle; TP = testosterone propionate; FT = flutamide.
[a]Data presented as mean ± SD.
[b]For the androgen antagonist assessment, all exposure groups were co-administered 0.4 mg/kg/day TP.
[c]Decrease in N due to animal removal.
[d]Statistical analysis performed by ANOVA. If significant, the means were subjected to Dunnett's test (compared to vehicle control) using JMP version 12.0.1. mean ± SD.
[e]Statistical analysis performed using Kruskal Wallace nonparametric analyses. If significant, the means were subjects to Dunn's test (compared to vehicle control).

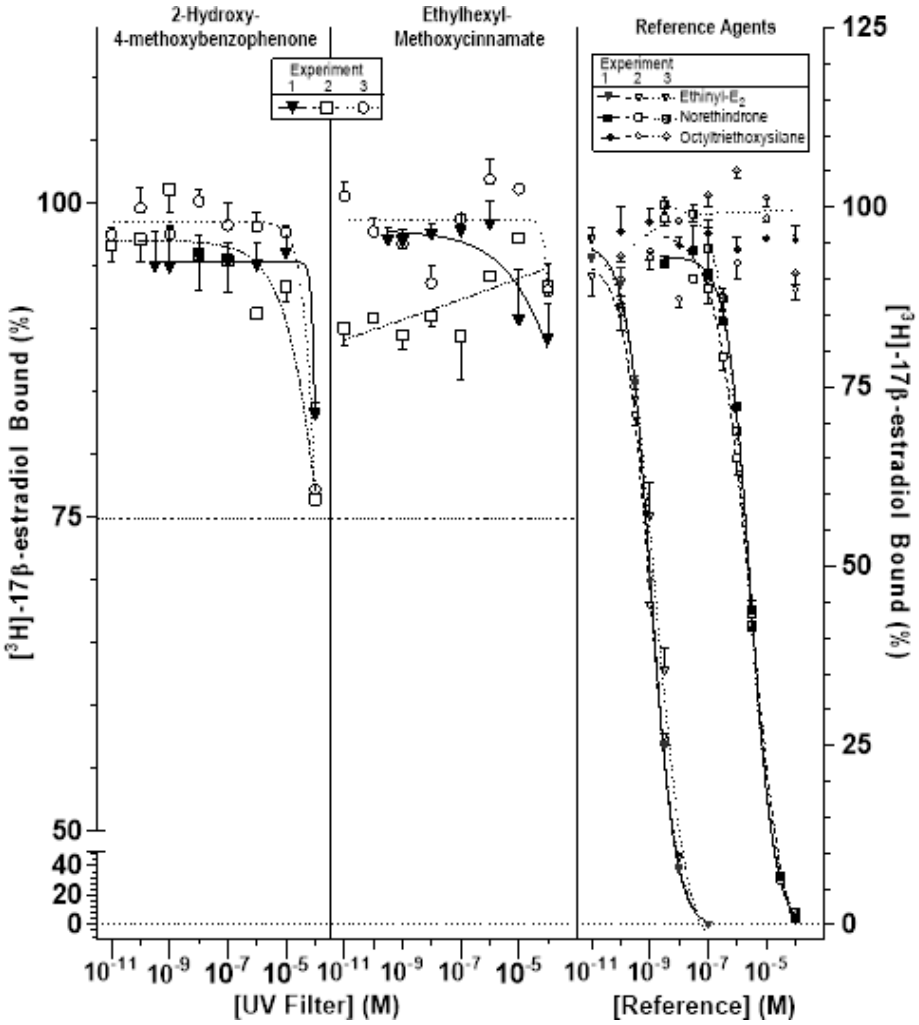

Figure F-1. Uterine Estrogen Receptor Binding

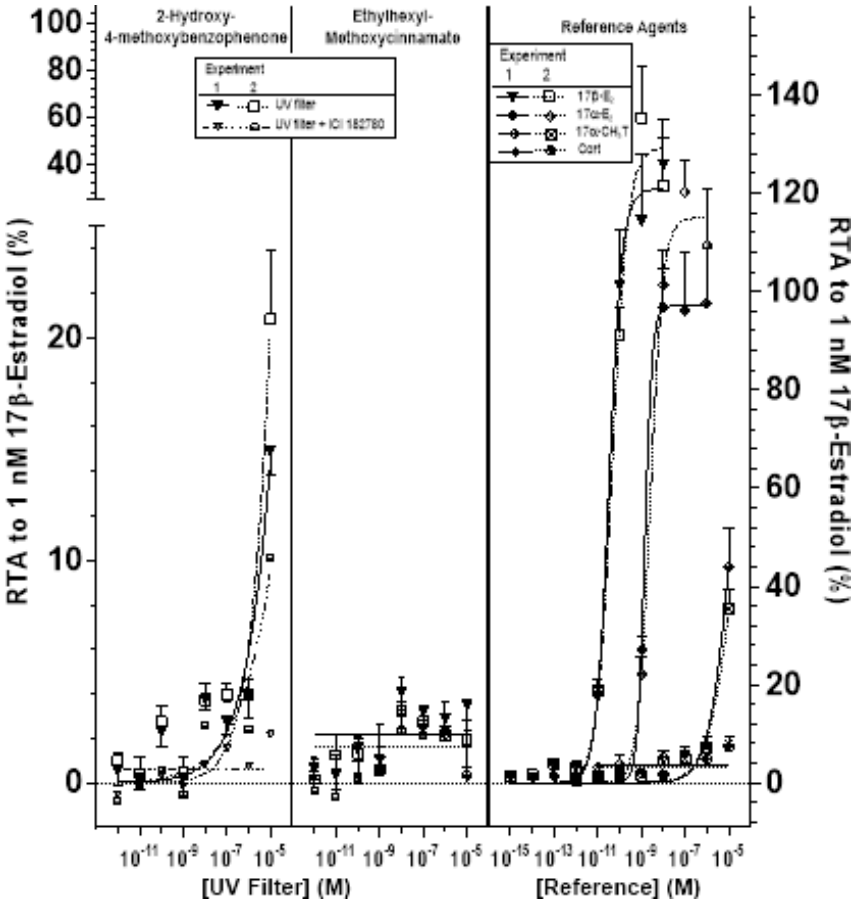

Figure F-2. Estrogen Receptorα Transcriptional Activation Assay

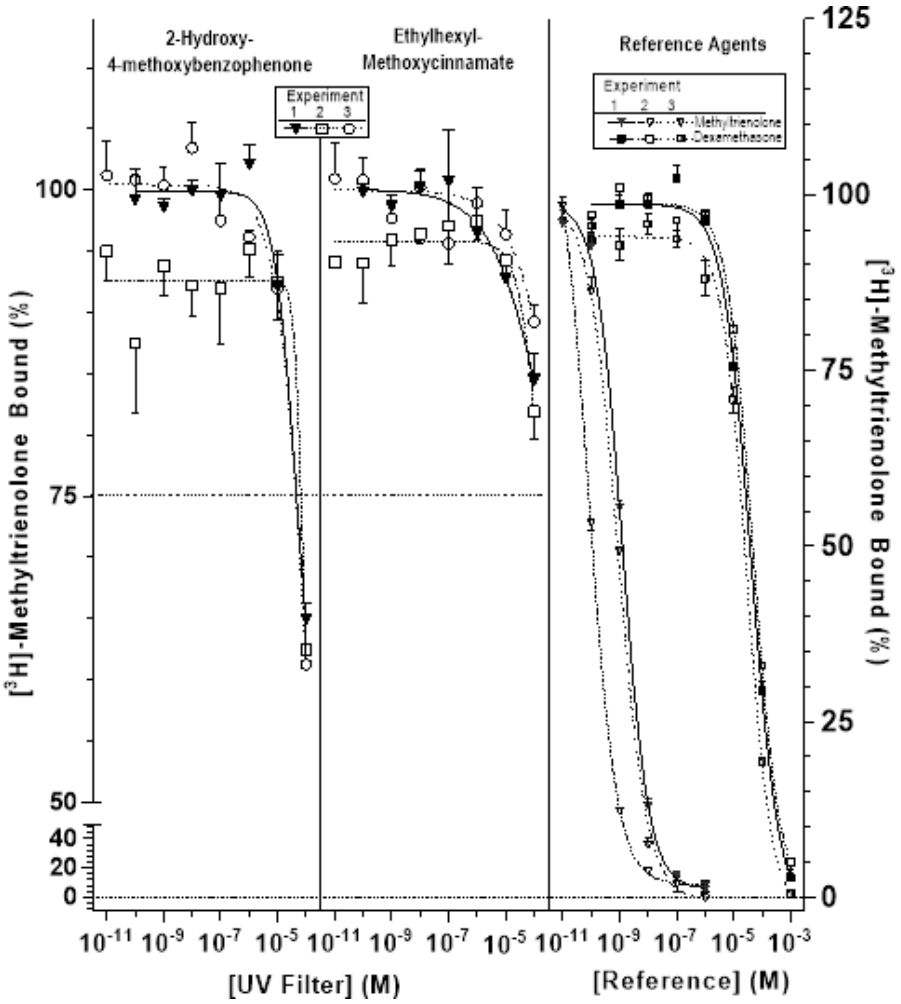

Figure F-3. Prostate Androgen Receptor Binding

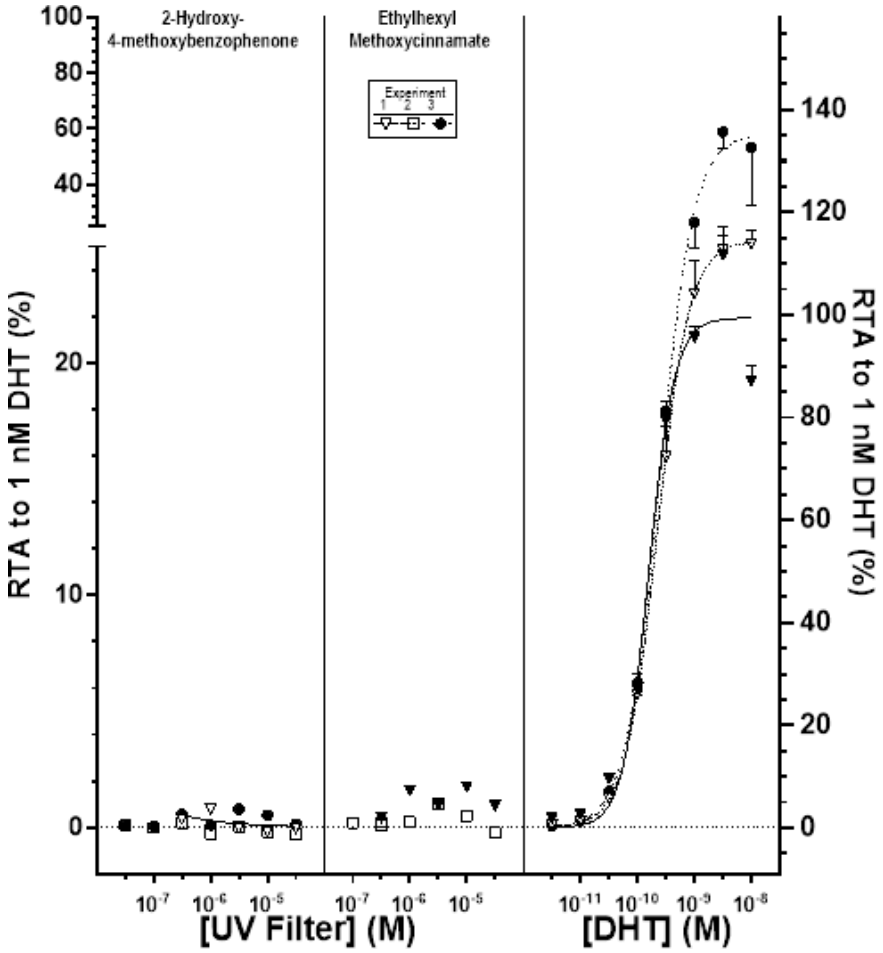

Figure F-4. Androgen Receptor Transactivation – Agonist Mode

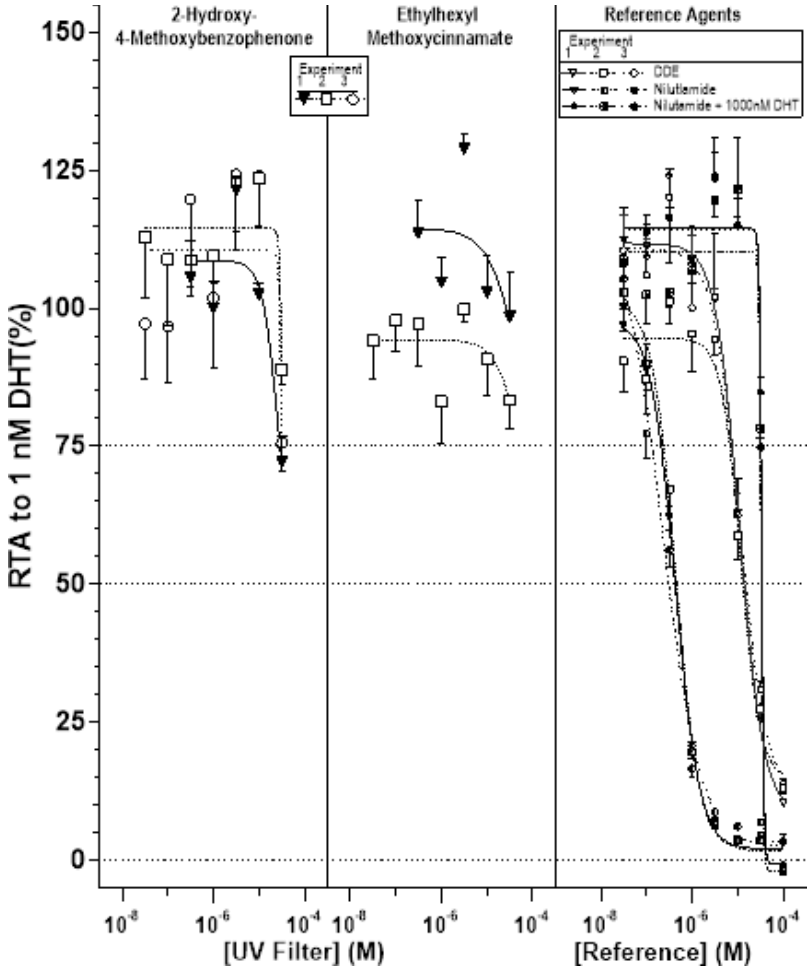

Figure F-5. Androgen Receptor Transactivation – Antagonist Mode

Appendix G. Summary of Peer Review Panel Comments

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G.1. Introduction

The National Toxicology Program (NTP) convened the NTP Technical Reports Peer Review Panel via webcast on December 12, 2019, to peer review the draft NTP Technical Reports on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone and Perfluorooctanoic Acid. Meeting information, including the draft reports, actions, and presentations is currently archived under NTP's "Past Events."

The panel peer reviewed the draft reports and provided its opinion on NTP's preliminary conclusions regarding the level of evidence of carcinogenic activity of 2-hydroxy-4-methoxybenzophenone and perfluorooctanoic acid. The panel's comments for the draft NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone begin at Section G.3.3. The panel's recommendations do not necessarily represent the opinion of NTP.

G.2. Attendees

Peer Review Panel Chair
Russell Cattley, Auburn University

Peer Review Panel
Michael Elwell, APEX TOXPATH, LLC
Wendy Halpern, Genentech, Inc.
Gabriele Ludewig, University of Iowa
Kristini Miles, Venture Chemical Consulting, LLC
Karen Regan, Regan Pathology/Toxicology Services, Inc. (2-hydroxy-4-methoxybenzophenone only)

National Institute of Environmental Health Sciences Staff
Brian Berridge
Chad Blystone
Michelle Cora
Stephen Ferguson
Shawn Harris
Angela King-Herbert
Ron Herbert
Dave Malarkey
Elizabeth Maull, Designated Federal Official
Barry McIntyre
Georgia Roberts
Sheena Scruggs
Keith Shockley
Robert Sills
Matthew Stout
Suramya Waidyanatha
Nigel Walker
AtLee Watson

Kristine Witt
Mary Wolfe
Other Federal Agency Staff
Gonçalo Gamboa, FDA (by WebEx)

Contract Support Staff
Amy Brix, EPL, Inc.
Canden Byrd, ICF
Josh Cleland, ICF
Katherine Duke, ICF
Sophie Hearn, ICF
Ernie Hood, Bridport Services
Kyathanahalli Janardhan, ILS
Kelly Shipkowski, ICF
Samantha Snow, ICF
Jessica Wignall, ICF

G.3. Peer Review of the Draft NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone and Perfluorooctanoic Acid

G.3.1. Introductions and Welcome

The National Toxicology Program (NTP) convened a peer-review panel for the *Draft NTP Technical Reports on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone* and *Perfluorooctanoic Acid* on December 12, 2019 via webcast.

- Dr. Russell Cattley, panel chair, called the meeting to order at 10:00 a.m., welcomed everyone to the meeting, asked all attendees to introduce themselves, and reviewed the peer-review meeting format for the panel and audience.
- Dr. Brian Berridge, NTP Associate Director, welcomed all participants to the meeting.
- Dr. Elizabeth Maull read the conflict of interest policy statement and briefed the attendees on meeting logistics.

G.3.2. Background and Charge to the Panel

Dr. Chad Blystone gave a brief presentation on NTP draft technical reports, including information about the levels of evidence for the potential carcinogenic activity of the chemicals tested. He also described the NTP's historical controls, which are categorized by route of exposure and rodent strains. He then gave the charge to the panel for the individual peer reviews:

- Review and evaluate the scientific and technical elements of the study and its presentation.
- Determine whether the study's experimental design, conduct, and findings support NTP's conclusions regarding the hypothesis under the conditions of this study. The peer-review meeting materials can be found on the NTP website.

G.3.3. Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone

G.3.3.1. Presentation and Clarifying Questions

Dr. Barry McIntyre summarized the studies and conclusions reported in the *Draft NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2-Hydroxy-4-methoxybenzophenone (CAS No. 131-57-7) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6C3F1/N Mice*.

2-Hydroxy-4-methoxybenzophenone is an ultraviolet (UV) filter used in sunscreens and cosmetics to protect the wearer from solar erythema. 2-Hydroxy-4-methoxybenzophenone is also an indirect food additive as it is added to acrylic and modified acrylic plastics that encounter food to prevent UV-mediated degradation. NTP chose to study 2-hydroxy-4-methoxybenzophenone due to widespread human exposure and lack of carcinogenicity data.

Dr. McIntyre first presented a summary of results from the 2-hydroxy-4-methoxybenzophenone Endocrine Disruptor Screening Panel studies reported in Appendix F of the technical report.

Genetic toxicology studies conducted in *Salmonella typhimurium* strains TA98 and TA100 as well as *Escherichia coli* strain *uvr*A pKM101 with and without S9 were negative.

NTP conducted a perinatal toxicity/carcinogenicity study in time-mated female Hsd:Sprague Dawley® SD® rats, with exposure concentrations of 0, 1,000, 3,000, and 10,000 ppm in the diet. Exposure began on gestation day (GD) 6 through lactation in the dams. In the offspring, exposure continued after weaning on postnatal day (PND) 21 (n = 50/dose) for 2 years. In addition, there was a 14-week interim necropsy for the 0 and 10,000 ppm groups (n = 10/sex/dose). Dr. McIntyre presented a summary of results from the 2-hydroxy-4-methoxybenzophenone perinatal toxicity/carcinogenicity study.

NTP also conducted a standard chronic bioassay in male and female B6C3F1/N mice, with exposure concentrations of 0, 1,000, 3,000, and 10,000 ppm in the diet for 2 years. Dr. McIntyre presented a summary of findings from the standard chronic bioassay.

Under the conditions of these 2-year studies, NTP's draft conclusions were:

- ***Equivocal evidence of carcinogenic activity*** of 2-hydroxy-4-methoxybenzophenone exposure in male Hsd:Sprague Dawley® SD® rats based on the occurrence of brain and spinal cord malignant meningiomas.
- Exposure to 2-hydroxy-4-methoxybenzophenone resulted in increased incidences of nonneoplastic lesions of the testis and pancreas in male rats.
- Equivocal evidence of carcinogenic activity of 2-hydroxy-4-methoxybenzophenone exposure in female Hsd:Sprague Dawley® SD® rats based on the increased incidence of thyroid C-cell adenomas and the increased incidence of uterine stromal polyps.
- Exposure to 2-hydroxy-4-methoxybenzophenone resulted in increased incidences of nonneoplastic lesions of the uterus and adrenal cortex in female rats.
- No evidence of carcinogenic activity in male or female B6C3F1/N mice at exposure concentrations of 1,000, 3,000, and 10,000 ppm 2-hydroxy-4-methoxybenzophenone.

- Exposure to 2-hydroxy-4-methoxybenzophenone resulted in increased incidences of nonneoplastic lesions of the bone marrow, spleen, and kidney in male and female mice, and liver in male mice.

There were no clarifying questions or comments about the presentation.

G.3.3.2. Public Comments

Dr. Cattley acknowledged the receipt of written public comments from private citizen Mr. Joe DiNardo. He noted that the panel did not receive requests for oral public comments on the draft technical report.

G.3.3.3. Peer-Review Comments and Panel Discussion

G.3.3.3.1. First Reviewer – Dr. Karen Regan

Dr. Karen Regan said that the studies were well-written and well-conducted. She requested clarification on how the dose used in the studies compared to human exposure. She asked for more information on the historical control data used for this study and whether there were any data available from the same strain of rat exposed from GD 6. Dr. Regan questioned why NTP did not evaluate spinal cords for all animals in the study. Dr. Regan asked whether there were any tables that showed the overall incidence of multiplicity for thyroid tumors in female rats, and whether there were any animals with multiple tumors.

- Dr. McIntyre indicated that the plasma levels found in the rodents following exposure were comparable to levels recently reported in human blood (JAMA 2019).
- Dr. Blystone reported that the historical controls used in this study include all exposure start times with most of these studies starting at GD 6.
- Dr. Amy Brix stated that only animals with clinical neuropathological signs had their spinal cords examined and indicated that NTP would consider adding a sentence to the report to clarify this point. She noted that it is possible that they did not observe additional occurrences of malignant meningiomas in the spinal cords. Dr. Brix noted that there were no occurrences of these type of tumors in the historical controls or control group. Even without the single occurrence in the spinal cord, NTP considered the occurrence of malignant meningioma in the brain of adult rats to be equivocal evidence of carcinogenicity.
- Dr. Brix noted that only one female in the highest dose group had bilateral C-cell adenomas in the thyroid, with the rest of the adenomas being unilateral. She remarked that NTP only counted an animal once if it had both a thyroid adenoma and carcinoma, which occurred in only one rat. She stated that they will explain these methods further in the report.

G.3.3.3.2. Second Reviewer – Dr. Kristini Miles

Dr. Kristini Miles stated that the study was well-designed and executed. She noted that NTP chose sufficient doses for the rats and mice as they were based on previously conducted studies. Dr. Miles noted that 2-hydroxy-4-methoxybenzophenone has been reported in the literature to be a persistent environmental contaminant, specifically in water sources, and there are reports that traditional wastewater treatment processes may not eliminate the contaminant. She asked whether NTP tested 2-hydroxy-4-methoxybenzophenone in the municipal tap water used in the

study. Dr. Miles inquired whether there was any information available on 2-hydroxy-4-methoxybenzophenone concentrations in synthetic resins and plastics, and how it might leach out of such containers. She asked that NTP include the information in the report. Regarding Table 8 in the report, Dr. Miles asked whether the trend test referred to historical controls or something else.

- Dr. McIntyre stated that they did not examine the municipal water used for the presence of 2-hydroxy-4-methoxybenzophenone; however, all rodents received the same water supply.
- Dr. McIntyre indicated that NTP provided information on leachates in plastics, and they would consider adding this information to the report.
- Dr. Keith Shockley noted that they based the trend test on experimental data, not historical controls.

G.3.3.3.3. Third Reviewer – Dr. Michael Elwell

Dr. Michael Elwell indicated that he had no comments on the study design or dose selection and the results and figures were clearly presented by the NTP. He concurred with the neoplastic and nonneoplastic findings listed in the draft report for the male rats. Regarding the data and discussions of the meningioma and interstitial cell hyperplasia, Dr. Elwell requested confirmation that NTP examined spinal cords from only one or two animals per group. He asked whether it was correct to combine the spinal cord and brain data as the study staff did in Table 11 to perform statistical analysis on the occurrence of these tumors. Dr. Elwell noted that there was one meningioma present in the spinal cord but three in the brain in male rats exposed to 1,000 ppm. He remarked that one of these animals (Animal 151) also had a meningioma recorded in the trigeminal nerve. He asked whether NTP should report that meningioma separately and include it in the discussion. He questioned whether NTP should mention the occurrence of granular cell tumors in the meninges in the report as part of the assessment of the meningiomas since NTP Technical Report 573 discussed the relationship between granular cell tumors and meningiomas, with an indication that they had similar morphologies and a common progenitor cell type. Dr. Elwell recommended that the NTP report the increased incidence of interstitial cell hyperplasia of the testes in the discussion section since they included it in the abstract and results section. Dr. Elwell agreed in principle with the conclusion of no evidence of carcinogenicity in the male mice. He suggested clarifying the wording that described the cytoplasmic alteration of the kidneys that occurred. Dr. Elwell stated that the incidence of lymphocyte infiltrates and nephropathy in the kidney listed in the abstract appeared to be very minor given the minimal severity and common background occurrence of these findings. Dr. Elwell remarked that prior studies with ethylbenzene reported no association of syncytial cell alterations in the liver with hepatocellular neoplasia. He suggested this would be worth mentioning in the discussion since ethylbenzene has a similar structure to 2-hydroxy-4-methoxybenzophenone.

In response to Dr. Elwell's comments, Dr. Brix indicated that:

- While NTP examined the brains of all animals, only animals with clinical neurological signs had their spinal cords examined. NTP will update the table to reflect this. NTP will also consider removing the spinal cord data from this analysis.

- There was an error in the table reporting two meningiomas in Animal 151 (one should have been recorded as a metastasis of the other) and they would update the table to reflect this. NTP will also consider adding language to the discussion section regarding malignant meningiomas.
- NTP will consider adding to the discussion section that meningiomas and granular cell tumors may have a common progenitor.
- NTP will consider adding text to the report that there was no effect on interstitial cell adenomas reported in male rats. NTP will also consider adding text to the discussion regarding interstitial cell hyperplasia.
- There was an error in describing the cytoplasmic alteration in the kidneys in male mice. NTP will update the report to reflect this.
- NTP will consider adding language to the results section downplaying the importance of the chronic progressive nephropathy and lymphocytic infiltrates in the kidneys of male mice and will remove mentions of these findings from the discussion.
- NTP would consider adding language to the report regarding the lack of association between syncytial cell alteration in the liver and exposure to ethylbenzene.

In a follow-up question, Dr. Elwell described his confusion regarding the statistical approaches for the non-neoplastic kidney findings in this report, which seemed to take severity into account.

Dr. Shockley replied that the statistical analysis did not take severity into account but was instead based on incidence. However, this method did account for differential survival.

G.3.3.4. Panel Discussion

Dr. Gabriele Ludewig stated that the report was clear and well-written. She wondered whether the number of incidences of meningiomas indicated a relationship to exposure and whether NTP should report this as clear evidence instead of equivocal. Dr. Ludewig asked for comment on why they used rats and not hamsters since they metabolize this compound differently, which may make it genotoxic. Dr. Ludewig noted that ER and AR binding is only one way a compound can be an endocrine disruptor. She noticed in the report that NTP reported findings in the adrenal gland which could lead to serotonin disruption and suggested adding that point to the report. Dr. Ludewig observed that only the highest exposure group had female rats that were not pregnant and suggested adding a statement regarding this point.

- Dr. Brix stated that it is always challenging to make a call on uncommon tumors without statistical support, dose response, a change in latency, or supporting evidence from other sex, species, or preneoplastic lesions. Therefore, NTP decided there was equivocal evidence based on the presence of meningiomas.
- Dr. McIntyre noted that NTP no longer used the hamster S9 assay under Organisation for Economic Co-operation and Development (OECD) guidance and that performing those tests would be outside the scope of this report.
- Dr. McIntyre indicated that NTP has done extensive work in relation to testing for hormonal activity and is in the process of reporting on reproductive performance as well as markers of altered endocrine action in another technical report.

- Dr. McIntyre noted that they examined the animals that did not deliver for implantation sites and they were not pregnant. Since exposure began after implantation, they were not concerned that this was an exposure-related effect.

In a follow-up statement, Dr. Ludewig concluded that she was between some and equivocal evidence regarding the presence of meningiomas.

Dr. Wendy Halpern noted that other reviewers addressed most of her comments. She asked the panel to comment on whether it was a common observation that there were fewer potentially exposure-related tumors identified at the highest dose where the animals had lower body weight.

- Dr. Blystone reported that the influence of lower body weight on the cancer response has been characterized for only a few tumor types (e.g., mammary gland tumors) and that we do not have the information for the other tumor types to include in the report.

Dr. Cattley questioned why there had been a separate peer review conducted on the uterus in the rats.

- Dr. Brix reported that over the past several years, NTP had changed its procedure from doing cross-sectional evaluations to more comprehensive analyses of the uterus.
- Dr. Brix reported that NTP would consider adding language to the report to clarify this point.

G.3.3.5. Vote on NTP Conclusions

G.3.3.5.1. Male Hsd:Sprague Dawley® SD® rats

Dr. Cattley called for a motion from the panel to approve the conclusions as written. The panel did not offer a motion. Dr. Regan moved that NTP delete the reference to spinal cord malignant meningiomas from the conclusion. Dr. Ludewig seconded the motion. The panel voted unanimously (5 yes, 0 no, 0 abstentions) to approve the new conclusion.

G.3.3.5.2. Female Hsd:Sprague Dawley® SD® rats

Dr. Cattley called for a motion from the panel to approve the conclusions as written. Dr. Regan so moved, and Dr. Elwell seconded the motion. The panel voted unanimously (5 yes, 0 no, 0 abstentions) to approve the conclusions as written.

G.3.3.5.3. Male B6C3F1/N mice

Dr. Cattley called for a motion from the panel to approve the conclusions as written. Dr. Regan so moved and Dr. Ludewig seconded the motion. The panel voted unanimously (5 yes, 0 no, 0 abstentions) to approve the conclusions as written.

G.3.3.5.4. Female B6C3F1/N mice

Dr. Cattley called for a motion from the panel to approve the conclusions as written. Dr. Regan so moved, and Dr. Ludewig seconded the motion. The panel voted unanimously (5 yes, 0 no, 0 abstentions) to approve the conclusions as written.

Following the voting, Dr. Cattley noted that Dr. Regan would sign off from the session.

G.3.4. Toxicology and Carcinogenesis Studies of Perfluorooctanoic Acid

G.3.4.1. Presentation and Clarifying Questions

Dr. Blystone summarized the studies and conclusions reported in the *Draft NTP Technical Report on the Toxicology and Carcinogenesis Studies of Perfluorooctanoic Acid (CAS No. 335-67-1) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats*.

Perfluorooctanoic acid is a perfluoroalkyl substance (PFAS) used for decades in creating non-stick properties in a variety of products. Manufacturers agreed to discontinue use due to widespread exposure and health concerns. Due to a long half-life measured in years and resistance to environmental degradation, exposure has continued but declined. Perfluorooctanoic acid is the second most abundant PFAS measured in the human population, including children and pregnant women.

Human exposure to perfluorooctanoic acid can occur during early development. It is unknown whether exposure during gestation and lactation alters the carcinogenic response induced by perfluorooctanoic acid. NTP tested the hypothesis that including perinatal exposure with postweaning exposure would quantitatively or qualitatively alter the perfluorooctanoic acid response compared to postweaning exposure only.

NTP conducted a perinatal and postweaning toxicity/carcinogenicity study in Hsd:Sprague Dawley® SD® rats. In Study #1, they exposed time-mated female rats to 0, 150, or 300 ppm perfluorooctanoic acid during the perinatal period. NTP provided F_1 female rats with 0, 300, or 1,000 ppm perfluorooctanoic acid during the postweaning period (i.e., perinatal/postweaning exposures of 0/0, 0/300, 0/1,000, 150/300, or 300/1,000 ppm) while they provided F_1 male rats with 0, 150, or 300 ppm perfluorooctanoic acid during the postweaning period (i.e., perinatal/postweaning exposures of 0/0, 0/150, 0/300, 150/150, or 300/300 ppm) (n = 50/sex/dose). Female rats have a lower systemic exposure than males due to a faster perfluorooctanoic acid elimination rate, so NTP provided a higher feed exposure concentration to female rats postweaning. In addition, they conducted a 16-week (19 weeks of age) interim necropsy (n = 10/sex/dose).

Due to observed unanticipated toxicity in males during the interim necropsy, NTP removed males from Study #1 at week 21. In Study #2, they exposed time-mated female rats to 0 or 300 ppm perfluorooctanoic acid during the perinatal period. They provided F_1 male rats 0, 20, 40, or 80 ppm perfluorooctanoic acid during the postweaning period (i.e., perinatal/postweaning exposures of 0/0, 0/20, 0/40, 0/80, 300/0, 300/20, 300/40, or 300/80 ppm) (n = 50/sex/dose). Dr. Blystone presented a summary of results from the perinatal and postweaning toxicity/carcinogenicity study.

Under the conditions of these 2-year studies, NTP's draft conclusions were:

- ***Clear evidence of carcinogenic activity*** of perfluorooctanoic acid in male Hsd:Sprague Dawley® SD® rats based on the increased incidences of hepatocellular neoplasms (predominately hepatocellular adenomas) and increased incidences of acinar cell neoplasms (predominantly acinar cell adenomas) of the pancreas.
- Exposure to perfluorooctanoic acid resulted in increased incidences of nonneoplastic lesions in the liver and pancreas of male rats. The additional effect of combined

perinatal and postweaning exposure was limited to a higher incidence of hepatocellular carcinomas compared to postweaning exposure alone.

- Some evidence of carcinogenic activity of perfluorooctanoic acid in female Hsd:Sprague Dawley® SD® rats based on the increased incidences of pancreatic acinar cell adenoma or adenocarcinoma (combined) neoplasms.
- The higher incidence of hepatocellular carcinomas and higher incidence of adenocarcinomas of the uterus may have been related to perfluorooctanoic acid exposure.
- Exposure to perfluorooctanoic acid resulted in increased incidences of nonneoplastic lesions in the liver, kidney, forestomach, and thyroid gland.
- The combined perinatal and postweaning exposure was not observed to change the neoplastic or nonneoplastic response compared to postweaning exposure alone.

There were no clarifying questions or comments about the presentation.

G.3.4.2. Public Comments

Dr. Cattley acknowledged the receipt of three written public comments from Dr. Oyebode A. Taiwo on behalf of the 3M Company, Dr. Alexis Temkin on behalf of the Environmental Working Group, and Mr. Jason Dadakis from the Orange County Water District. Dr. Cattley noted that there was one oral public comment from Mr. Steve Risotto on behalf of the American Chemistry Council (ACC).

Mr. Risotto said the ACC believed the peer-review committee should carefully consider NTP's conclusion that there is "some evidence of carcinogenic activity" in female rats, requested additional analysis of the pancreatic tumor data for male rats, and asked whether a conclusion could be reached about the sensitivity of fetal rats to perfluorooctanoic acid exposure. Regarding the evidence for carcinogenicity in female rats, Mr. Risotto observed that there was a non-significant increase of combined acinar cell adenomas and adenocarcinomas at 1,000 ppm. He noted that while the increased incidence of acinar cell neoplasms in males increased NTP's confidence that neoplasms in females were related to perfluorooctanoic acid exposure, NTP did not observe acinus hyperplasia in the female rats, which was significantly increased in the male rats. If this hyperplasia is a potentially preneoplastic lesion as NTP suggests, Mr. Risotto stated that this finding should also have been observed in the female rats. Mr. Risotto also noted that the survival rate in the female rats was quite low, which might raise concerns about the general animal husbandry practices of the study since survival was depressed in both the control and exposed animals. Regarding the incidence of acinar cell neoplasms in the male rats, Mr. Risotto noted that the control group had significantly elevated acinus hyperplasia, a possible preneoplastic lesion, affecting nearly 40% of the control animals. The high background rate observed in the study confirmed the higher sensitivity of the Hsd:Sprague Dawley® SD® rats compared to other rat strains, and more significantly, to humans. Mr. Risotto also observed that NTP used a smaller size criterion for classifying pancreatic acinar cell neoplasms than previous perfluorooctanoic acid studies. He stated that the draft report does not provide an explanation for why NTP reduced the lesion criteria, or the potential impact such a reduction may have on the findings. Mr. Risotto stated that given the flat dose response for acinar cell neoplasms and high rate of preneoplastic hyperplasia in the control group, NTP should further consider the pancreatic results in the male rats, particularly given the likely contribution of peroxisome proliferator-

activated receptor alpha (PPARα) to tumor formation. Mr. Risotto questioned the relevance of the male rat findings to human risk assessment. He said that ACC was unable to find additional information in the draft report on the nature of the toxicity that caused NTP to restart the male portion of the study. He added that it is critically important that the committee understand the nature of the unanticipated toxicity and consider its potential significance to the findings in the draft report. Regarding the conclusion about fetal sensitivity to perfluorooctanoic acid exposure, Mr. Risotto observed that the draft report indicates that there were very few significant differences between the groups of animals exposed postweaning-only versus groups with perinatal and postweaning exposure. He noted that the differences observed were sporadic and that the report does not prove a conclusion regarding the potential impact of perinatal exposure yet that is the stated hypothesis for conducting the study. Given that NTP rarely conducts combined perinatal and postweaning chronic studies, Mr. Risotto said that ACC believes the committee should evaluate whether the study results support NTP's central hypothesis. He stated that the report summary suggests NTP does not support the hypothesis.

G.3.4.3. Peer-Review Comments and Panel Discussion

G.3.4.3.1. First Reviewer – Dr. Michael Elwell

Dr. Elwell said that both studies were well-designed, nicely presented, and NTP clearly stated the results. Dr. Elwell requested further discussion of why the proposed level of carcinogenic activity for pancreatic neoplasms in females does not also apply to the liver carcinoma findings. He said the rationale for the pancreas acinar neoplasm increases as "some evidence" in females appears to be based on the current and historical control data, and association with the finding of "clear evidence" in males. He felt that the rationale could apply to the female liver neoplasm conclusion as well. Dr. Elwell requested discussion of why NTP did not consider the uterine carcinomas "equivocal." Dr. Elwell asked about the relationship of the extended and standard evaluations on the incidence of adenoma and adenocarcinoma in the uterus.

In response to Dr. Elwell's comments, Dr. Blystone indicated that:

- NTP applied a call of *some evidence of carcinogenic activ*ity to the adenomas and adenocarcinomas in the pancreas in females since they were present in the highest dose group and considered a rare lesion. However, in the liver, there was only a marginal increase in the incidence of hepatocellular carcinomas in females and no change in the incidence of adenomas.
- NTP used a weight of evidence approach to determine that uterine adenocarcinomas might have been related to exposure.
- NTP found most of the uterine adenocarcinomas after the new sectioning and extended evaluation.

G.3.4.3.2. Second Reviewer – Dr. Gabriele Ludewig

Dr. Ludewig agreed that NTP executed and described the studies very well. She noted that due to the interest in the results for human risk assessment, the introduction should be perfectly clear as to the limitations of the study. Dr. Ludewig suggested that NTP should clearly state the reference to the perfluorooctanoic acid half-life in female rats, which is measured in hours, versus the half-life in humans, which is measured in years, in the introduction to prevent a false assumption of safety from the high dose that was used. Dr. Ludewig recommended clarifying what type of

"overt toxicity" NTP observed in males in Study #1. Dr. Ludewig suggested they add a statement to the discussion as to whether they considered/analyzed ossification and changes in bone morphology since regulatory values in the United States are based on these findings. Dr. Ludewig recommended emphasizing in the report that the PPARα pathway, which NTP mentions in the report, is not relevant to humans. The CAR pathway and any observation of its activation should be discussed. Dr. Ludewig said NTP should clearly state that the adult exposure included some developmental exposure during sexual maturation since the rats do not fully develop until PND 90. She noted this is important since the hypothesis was to test whether there was an influence of developmental exposure. Dr. Ludewig pointed out that NTP did not mention fecal excretion. Dr. Ludewig stressed that they clearly state the study limitations, especially since the short half-life in rodents is thought to be due to specific transporters present in the kidneys and other organs which may have influenced organ-specific concentrations of PFOA. Dr. Ludewig reported that NTP should cite recently published studies on perfluorooctanoic acid, glucose levels, and liver toxicity to support the findings that there is some liver toxicity.

- Dr. Blystone indicated that NTP would consider clarifying some of the language and adding statements to the report based on her suggestions. This may include additional information on the differences in perfluorooctanoic acid elimination between rodents and humans, a statement about bone morphology findings, and highlighting the point that exposure occurred during sexual maturation.

G.3.4.3.3. Third Reviewer – Dr. Wendy Halpern

Dr. Halpern indicated that NTP clearly presented and described the findings in the draft report. Dr. Halpern suggested adding more information to the discussion section, in a similar manner to the introduction, that highlights the potential mechanisms of these findings. For instance, the discussion could address the fact that previous carcinogenicity studies identified interstitial cell tumors in male rats as potentially related to perfluorooctanoic acid exposure, which NTP did not identify in this study. Dr. Halpern requested more discussion on the potential immunomodulatory activity as indicated by the decreased spleen and thymus weights. Dr. Halpern suggested expansion of the interim necropsy results, particularly the effect on liver weights. She noted that the biggest challenge in interpreting the study results is the exposure differences, both between rats and humans and between male and female rats. For instance, if testosterone is the driver for exposure differences in males, it is hard to interpret the exposure relationship during development when testosterone is still low in male pups and where female pups were given a higher dose based on exposure differences in adult rats. Dr. Halpern agreed that the decreased body weight and hepatic findings in the males in the initial 16-week necropsy warranted a change in dose administration. However, it is difficult to distinguish the sex-related from potential study-related differences in hepatocellular carcinoma incidence, especially with a small absolute magnitude of difference. Dr. Halpern noted that in almost every parameter evaluated, perinatal exposure seemed to have minimal or no effect, with the singular exception of a numerical increase in hepatocellular carcinomas in male rats exposed to 300/80 ppm in Study #2. She did not think the overall data supports that perinatal exposure drives this finding since there was a numerically similar incidence of hepatocellular carcinomas in the female rats and because of the potential linkage of these neoplasms to PPARα. She noted that she did not see a difference with perinatal exposure in either liver weights, acyl-CoA activation, or any other parameter that would suggest that PPARα induction was different. She did not think this finding was clearly

related or dependent on perinatal exposure, which goes back to the hypothesis presented. Dr. Halpern indicated that her main question was whether this was a developmentally sensitive effect given that male rats had the same exposure as female rats based on the kinetic data and these findings were not present in female rats.

In response to Dr. Halpern's comments, Dr. Blystone indicated that:

- NTP phrased the conclusion statements as they were because they limited the response to that one finding and, since perinatal exposure may by an influence, it was not an outright rejection of the hypothesis.
- NTP did not observe differences in liver weights or acyl-CoA oxidase activity. He noted that there might be other mechanisms at play besides increased PPARα via acyl-CoA activation, such as the constitutive androstane receptor (CAR) pathway. While CAR activity has been shown in other studies, NTP did not evaluate it in this study.
- NTP would consider clarifying various points in the report based on Dr. Halpern's suggestions such as the exposure concentration differences between this study and concentrations used previously, the role sexual maturation may play in the exposure differences, and details regarding spleen and thyroid weights.

In a follow-up statement, Dr. Halpern said it was her impression from reading the report that NTP did not expect that male and female perinatal exposures would have differed, yet they only saw hepatocellular carcinomas in the males, not the females.

- Dr. Blystone confirmed Dr. Halpern's statement.

G.3.4.4. Panel Discussion

Dr. Cattley expressed concern that there was not sufficient evidence to conclude that there was an influence of perinatal exposure. For instance, the hepatocellular carcinoma response was so weak that there was not much of a window to detect any increase that might occur without any perinatal exposure. In addition, there was no effect on the incidence of hepatocellular adenomas with perinatal exposure. He noted that the report appropriately combined the incidence of hepatocellular adenomas and hepatocellular carcinomas since NTP considered adenomas to be the precursor lesion and can potentially progress to carcinomas. He indicated that he was struggling with the hypothesis about the impact of perinatal exposure on the incidence of hepatocellular neoplasia in the study.

- Dr. Blystone confirmed that the incidence of adenomas did not appear different between animals with and without perinatal exposure. He remarked that although the strength of response was not strong, the rarity of the lesion and the fact that the liver responded to the chemical led NTP to make this conclusion.

In a follow-up statement, Dr. Cattley indicated that he was still hesitant to conclude that there was a perinatal effect in this study.

G.3.4.5. Vote on NTP Conclusions

G.3.4.5.1. Male Hsd:Sprague Dawley® SD® rats

Dr. Cattley called for a motion from the panel to approve the conclusions as written. Dr. Ludewig so moved, but there was no second to the motion. Dr. Cattley called for a motion from the panel to approve the first two bullets as written and delete the third bullet, and the panel would consider adding a replacement third bullet. Dr. Halpern so moved and Dr. Ludewig seconded the motion. The panel voted unanimously (4 yes, 0 no, 0 abstentions) to approve the first two bullets of the conclusion. Dr. Cattley called for a motion to add a third bullet. Following an extensive discussion, the panel proposed the third bullet read, "The additional effect of perinatal exposure in combination with postnatal exposure was uncertain and limited to the observation of hepatocellular carcinomas." Dr. Halpern moved for the approval of the third bullet. Dr. Ludewig seconded the motion. The panel voted unanimously (4 yes, 0 no, 0 abstentions) to approve the proposed third bullet.

G.3.4.5.2. Female Hsd:Sprague Dawley® SD® rats

Dr. Cattley called for a motion from the panel to approve the conclusions as written. Dr. Elwell so moved and Dr. Ludewig seconded the motion. The panel voted unanimously (4 yes, 0 no, 0 abstentions) to approve the conclusions as written.

G.3.5. Closing Remarks on the Draft Reports

Dr. Cattley welcomed additional panel comments on the draft reports. There were no additional comments.

Closing the meeting, Dr. Maull thanked all the peer-review panelists.

Dr. Cattley added his thanks to the NTP staff and the panel members for their efforts.

Dr. Cattley adjourned the meeting at 1:15 p.m. EDT on December 12, 2019.

Appendix H. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-TR-597.

H.1. Fourteen-week Interim Evaluation in Rats

E03 – Growth Curves

E04 – Mean Body Weights and Survival Table

E05 – Clinical Observations Summary

E08 – Feed Water and Compound Consumption Table

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site

P04 – Neoplasms by Individual Animal

P05 – Incidence Rates of Neoplasms by Anatomic Sites (Systematic Lesions Abridged)

P08 – Statistical Analysis of Primary Tumors

P09 – Non-Neoplastic Lesions by Individual Animal

P10 – Statistical Analysis of Non-Neoplastic Lesions

P11 – Statistical Analysis of Survival Data

P14 – Individual Animal Pathology Data

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades

P40 – Survival Curves

PA06 – Organ Weights Summary

R02 – Reproductive Performance Summary

H.2. Perinatal and Two-year Study in Rats

E03 – Growth Curves

E04 – Mean Body Weights and Survival Table

E05 – Clinical Observations Summary

E08 – Feed Water and Compound Consumption Table

HMB Chronic Perinatal Female Harlan Sprague Dawley Rats Gestational and Lactational Chemical Consumption (mg HMB/kg body weight/day)HMB Chronic Perinatal Female Harlan Sprague Dawley Rats: Gestational and Lactational Food Consumption (g/animal/day)

HMB Chronic Perinatal Female Harlan Sprague Dawley Rats: Lactational Body and Body Weight Gains

HMB Chronic Perinatal Litter Data Analysis in Female Harlan Sprague Dawley Rats: Live Litter Size

HMB Chronic Perinatal Litter Data Analysis in Female Harlan Sprague Dawley Rats: PND1 Data

HMB Chronic Perinatal Litter Data Analysis in Female Harlan Sprague Dawley Rats: Survival

HMB Chronic Perinatal: Summary Pup Body Weights

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site

P04 – Neoplasms by Individual Animal

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)

P08 – Statistical Analysis of Primary Tumors with Rao-Scott

P09 – Non-Neoplastic Lesions by Individual Animal

P10 – Statistical Analysis of Non-Neoplastic Lesions

P11 – Statistical Analysis of Survival Data

P14 – Individual Animal Pathology Data

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades

P40 – Survival Curves

H.3. Two-year Study in Mice

E03 – Growth Curves

E04 – Mean Body Weights and Survival Table

E05 – Clinical Observations Summary

E08 – Feed Water and Compound Consumption Table

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site

P04 – Neoplasms by Individual Animal

P05 – Incidence Rates of Neoplasms by Anatomic Sites (Systemic Lesions Abridged)

P08 – Statistical Analysis of Primary Tumors

P09 – Non-Neoplastic Lesions by Individual Animal

P10 – Statistical Analysis of Non-Neoplastic Lesions

P11 – Statistical Analysis of Survival Data

P14 – Individual Animal Pathology Data

P17 –Neoplasms by Individual Animal (Systemic Lesions Abridged)

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades

P40 – Survival Curves

H.4. Genetic Toxicology

G06 – Ames Summary Data

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