OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

WASHINGTON, D.C. 20460

MEMORANDUM

DATE: July 2, 2024

SUBJECT: **Piperonyl Butoxide:** Second Report of the Cancer Assessment Review Committee

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CAS No.: 51-03-6
Decision No.: 579590
Petition No.: N/A
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FROM: Alexandra Turley, Ph.D., Executive Secretary
Cancer Assessment Review Committee
Health Effects Division (HED; 7509T)

THRU: Cynthia Browning, Ph.D., Co-Chair
Sarah Dobreniecki, Ph.D., Co-Chair
Cancer Assessment Review Committee
Health Effects Division (HED; 7509T)

TO: Jessica Kidwell, Biologist
Shalu Shelat, Branch Chief
Risk Assessment Branch IV
Health Effects Division (HED; 7509T)

and

Kaitlin Saunders, Biologist
Elizabeth Fertich, Product Manager 4
Invertebrate-Vertebrate Branch I (IVB1)
Registration Division (RD; 7505T)

The conclusions conveyed in this assessment were developed in full compliance with *EPA Scientific Integrity Policy for Transparent and Objective Science*, and EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions.* The full text of *EPA Scientific Integrity Policy for Transparent and Objective Science*, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/system/files/documents/2023-12/scientific_integrity_policy_2012_accessible.pdf. The full text of the EPA Scientific Integrity Program's *Approaches for Expressing and Resolving Differing Scientific Opinions* can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions.

HED's Cancer Assessment Review Committee (CARC) met on April 30th and May 2nd, 2024, to re-evaluate the carcinogenic potential of Piperonyl Butoxide (PBO) in accordance with the *EPA's Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the Cancer Assessment Document.

CANCER ASSESSMENT DOCUMENT

Piperonyl Butoxide (PBO)

PC Code 067501

Date of the Report: 07/02/2024

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

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I. EXECUTIVE SUMMARY

The Cancer Assessment Review Committee (CARC) met on April 30th and May 2nd, 2024, to re-evaluate the carcinogenic potential of Piperonyl Butoxide (PBO) and to determine a cancer classification in accordance with the EPA's *Guidelines for Carcinogen Risk Assessment* (March 2005). Previously, the Carcinogenicity Peer Review Committee (CPRC) classified PBO under the 1986 *Guidelines for Carcinogen Risk Assessment* as a *Group C - Possible Human Carcinogen*, based on liver tumors in both sexes of CD-1 mice, and recommended that for the purpose of risk characterization, the Reference Dose (RfD) and Margin of Exposure (MOE) approaches should be used for quantitation of human risk (J. Doherty and E. Rinde, TXR 0011576, 06/07/1995).

For the 2024 meetings, the CARC re-evaluated the mouse and rat carcinogenicity data (MRIDs 42903701 and 40323701, respectively) and the newly submitted data to support the PBO Task Force II's (PBTFII's) proposed mode of action (MOA) for liver tumors in male and female mice (MRIDs 51692500, 51692501, 51692502, 51692503, 51692504, 51692505, 51692506, 51692507, 51692508, 52376008). The CARC also re-considered additional carcinogenicity studies in rats and mice, identified mainly from the open literature, that were included in the previous cancer peer review assessment (MRIDs 52376001, 52376002, 52376006, 52376009, 52376010).

The CARC considered the following in its WOE deliberation in assessing the carcinogenic potential of PBO:

Mice

In a carcinogenicity study (MRID 42903701*)*, groups of 60 CD-1® mice/sex were administered PBO (90.78% a.i.; Lot # FEP-100 12/12/89) via the diet at dose levels of 0 (Group 1), 0 (Group 2), 30, 100, or 300 mg/kg/day for up to 78 weeks.

Liver Tumors

- **The CARC determined that the combined hepatocellular adenomas and/or carcinomas, driven by adenomas, seen at ≥100 mg/kg/day in male CD-1 mice are treatment related.** This was based on significant increasing trends in hepatocellular adenomas and combined adenomas and/or carcinomas, as well as significant differences in the pair-wise comparisons of the 100 and 300 mg/kg/day dose groups with the controls for hepatocellular adenomas and combined adenomas and/or carcinomas, all at $p<0.01$. For carcinomas, there was a significant increasing trend and significant difference in the pair-wise comparison of the 300 mg/kg/day dose group, both at $p<0.01$. The incidences of liver adenomas at 100 and 300 mg/kg/day were outside the historical control ranges of both the conducting laboratory and Charles River Laboratories. The incidence of liver carcinomas at 300 mg/kg/day was just outside the historical control ranges from both the conducting laboratory and Charles River Laboratories. Supporting pre-neoplastic lesions (liver hyperplasia and eosinophilic foci) were seen at 300 mg/kg/day at study termination. While the CARC relied primarily on the tumor data as reported in MRID 42903701, it also considered a 1995 pathology re-evaluation of the liver tumors (MRID 51692508). The

overall conclusion, however, was the same--that the combined liver tumors, driven by adenomas, were treatment related.

- **The CARC determined that the hepatocellular adenomas seen at 300 mg/kg/day (highest dose tested) in female CD-1 mice were treatment related.** Female mice had a significant increasing trend, and a significant difference in the pair-wise comparison of the 300 mg/kg/day dose group with the controls for adenomas, both at $p<0.01$. There were no liver carcinomas reported in the females. The incidence of liver adenomas at 300 mg/kg/day was outside the historical control ranges from both the conducting laboratory and Charles River Laboratories. Supporting pre-neoplastic lesions (liver hyperplasia and eosinophilic foci) were seen at 300 mg/kg/day at study termination.

- The CARC concluded that dosing in the mouse carcinogenicity study was adequate and not excessive to assess carcinogenicity. This was based on liver histopathology (eosinophilic foci and hyperplasia) seen at 300 mg/kg/day in males and at ≥100 mg/kg/day in females. Liver hypertrophy was also seen in males at ≥100 mg/kg/day and in females at 300 mg/kg/day. Increased liver weights were seen in both sexes at ≥100 mg/kg/day. Survival was not affected.

Rats

In a combined chronic toxicity/carcinogenicity study (MRID 40323701), groups of 60 Sprague-Dawley Crl:CDR (SD)BR rats/sex were administered PBO (87.67-89.71% a.i.; Reference # FEG32) in the diet at target dose levels of 0 (Group 1 control), 0 (Group 2 control), 30, 100, or 500 mg/kg/day for up to 104/105 (males/females) weeks.

Liver Tumors

- **The CARC concluded that the combined hepatocellular adenomas and/or carcinomas seen at 500 mg/kg/day in male Sprague Dawley (SD) rats and the hepatocellular carcinomas in female SD rats were not treatment related.** For males, this was based on significant increasing trends only in adenomas at $p<0.01$ and combined adenomas and/or carcinomas at $p<0.05$, but no significant differences in the pair-wise comparisons of adenomas, carcinomas or combined compared to controls at any dose. For females, the incidences of liver carcinomas were not biologically or statistically significant at any dose. There were no liver adenomas reported for females. For both sexes, the incidences of liver adenomas and/or carcinomas were within or just outside the historical control ranges for both the conducting laboratory and Charles River Laboratories.

Thyroid Tumors

- **The CARC concluded that the combined thyroid follicular cell adenomas and/or carcinomas seen in male SD rats and the thyroid follicular cell adenomas seen in female SD rats are not treatment related.** For males, this was based on a significant increasing trend only in thyroid follicular cell combined adenomas and/or carcinomas at $p < 0.05$, but no significant differences seen in the pair-wise comparisons of any dosed group with the controls for thyroid follicular cell

adenomas, carcinomas or combined. In addition, there was a lack of a dose response at the lower doses. For males, the incidences of thyroid follicular cell adenomas or carcinomas alone at 500 mg/kg/day were within the Charles River historical control ranges. The incidences of thyroid follicular cell combined adenomas and/or carcinomas at 500 mg/kg/day are just above the conducting laboratory historical control range. For females, a significant increasing trend only in thyroid follicular cell adenomas was seen at $p<0.05$, but there were no significant differences in the pair-wise comparisons of the dosed groups with the controls. There were no thyroid follicular cell carcinomas reported in the female rats. The incidences of adenomas in females were within the historical control ranges of both the conducting laboratory and Charles River Laboratories.

- The CARC concluded that dosing in the chronic toxicity/carcinogenicity study in rats was considered adequate, and not excessive, to assess carcinogenicity. This was based on treatment-related thyroid histopathology (thyroid follicular cell hyperplasia at ≥100 mg/kg/day in females), decreased body weight (both sexes) at 500 mg/kg/day, increased liver weight, clinical pathology (increased cholesterol), and macroscopic (focal mixed cells) and microscopic pathology (hypertrophy) findings in the liver at 500 mg/kg/day (both sexes). No statistically significant increases in mortality were seen for either sex compared to controls. The CARC concluded that the mortality noted in males at the end of the study did not compromise the integrity of the study overall and still allowed for valid statistical comparisons of tumors.

Mutagenicity

The CARC concluded that the overall weight of the evidence did not show a mutagenic concern for PBO based on a battery of genotoxicity assays.

Structure Activity Relationship (SAR)

There is limited SAR support which identified other synergists, including MGK-264, piperonyl sulfoxide, and safrole, with structural similarities of <80%, which were also potentially carcinogenic.

Mode of Action

The registrant proposed a mitogenic MOA for liver tumors involving constitutive androstane receptor (CAR) activation in male and female mice following PBO treatment. The key and associative events include CAR activation, evaluated indirectly through the induction of Cyp2b expression and Cyp2b enzyme activity, hepatic Cyp2b10 mRNA levels, microsomal 7-pentoxyresorufin *O*-dependentylase (PROD) activity and Cyp2b protein content (key event #1), increased cell proliferation (key event #2), increased liver weight (associative event #1), increased liver hypertrophy (associative event #2), clonal expansion leading to altered hepatic foci (key event #3), and liver tumors (key event #4).

Based on the evidence presented, the CARC concluded that, overall, there is strong concordance between key (and associative) events and the dose levels that produce tumors. The CARC also concluded that the key and associative events occur in a logical, time-dependent manner consistent with the sequence of events of the proposed CAR MOA. It is noted that the MOA data were conducted

in male mice only since the males were more susceptible to PBO-induced liver tumor formation than female mice. The CARC considered that information gathered using just one sex that is representative of the responses in both males and females was sufficient and does not detract from support for the overall MOA.

The CARC determined that likelihood that alternative MOAs for liver tumors are operative in mice has been adequately ruled out.

Consistent with current HED policy and practice, the CARC considers the CAR MOA for PBO induced mouse liver tumor formation to be qualitatively and quantitatively plausible in humans.

Overall, the CARC concluded that the WOE adequately supports a CAR-mediated mitogenic MOA for PBO-related liver tumors in male and female mice.

Classification of Carcinogenic Potential

In accordance with the EPA's *Guidelines for Carcinogen Risk Assessment* (March, 2005), the CARC classified PBO as "Not likely to be carcinogenic to humans at doses that do not induce cellular proliferation in the liver." This classification was based on the following WOE considerations:

1. Treatment-related increases in combined liver adenomas and/or carcinomas, driven by adenomas, were observed in male CD-1 mice at ≥100 mg/kg/day and treatment related increases in liver adenomas were observed in female CD-1 mice at 300 mg/kg/day.
2. The liver tumors and thyroid follicular cell tumors seen in male and female Sprague Dawley rats at 500 mg/kg/day were not considered to be treatment-related.
3. The mechanistic data sufficiently support the proposed CAR-mediated mitogenic MOA for liver tumors observed in male and female mice after PBO treatment.
4. There is no concern for the mutagenicity of PBO.
5. There is limited SAR support which identified other synergists, including MGK-264, piperonyl sulfoxide, and safrole, with structural similarities of <80%, which were also potentially carcinogenic.

Quantification of Carcinogenic Potential

Based on this cancer classification, quantification of cancer risk is not required. A non-linear approach (i.e., Reference Dose (RfD)) would adequately account for all the chronic toxicity, including carcinogenicity, that could result from exposure to PBO. The RfD should be protective of the dose (30 mg/kg/day) which induced hepatocellular proliferation in mice.

II. INTRODUCTION

Previously, the Carcinogenicity Peer Review Committee (CPRC) classified PBO under the 1986 *Guidelines for Carcinogen Risk Assessment* as a *Group C - Possible Human Carcinogen*, based on liver tumors in both sexes of CD-1 mice, and recommended that for the purpose of risk characterization, the Reference Dose (RfD) and Margin of Exposure (MOE) approaches should be used for quantitation of human risk (J. Doherty and E. Rinde, TXR 0011576, 06/07/1995).

The Cancer Assessment Review Committee (CARC) met on April 30th and May 2nd, 2024, to re-evaluate the carcinogenic potential of PBO and to determine a cancer classification in accordance with the *EPA's Guidelines for Carcinogen Risk Assessment* (March 2005). The committee re-evaluated the mouse and rat carcinogenicity data (MRIDs 42903701 and 40323701, respectively) and the newly submitted data to support the PBO Task Force II's (PBTFII's) proposed MOA for liver tumors in male and female mice (MRIDs 51692500, 51692501, 51692502, 51692503, 51692504, 51692505, 51692506, 51692507, 51692508). In addition, the CARC re-considered additional carcinogenicity studies in rats and mice, mainly from the literature, that were used by the previous cancer peer review in the overall WOE evaluation (MRIDs 52376001, 52376002, 52376006, 52376009, 52376010).

III. BACKGROUND INFORMATION

Piperonyl butoxide [5-[[2-(2-butoxyethoxy)ethoxy]methyl]-6-propyl-1,3-benzodioxole] (PBO) is a synergist used in combination with a wide variety of insecticides such as pyrethrins, allethrins, permethrin, tetramethrin, rotenone, and carbamates. Synergists are chemicals that lack pesticidal properties of their own but enhance the pesticidal effects of other active ingredients.

Registered PBO products contain 0.3% – 60% of active ingredient and are formulated as emulsifiable concentrates (EC) and dusts (D), as well as a number of ready to use (RTU) products. Commercial uses of PBO include pre- and post-harvest applications to food and non-food agricultural crops; applications in food and non-food handling commercial and agricultural structures and outdoor premises; in housing for veterinary and farm animals; and direct applications to veterinary and farm animals. Residential uses of PBO include pest control in homes and outdoor domestic structures, on gardens, lawns, ornamentals, and direct application to household pets.

Applications can be made to agricultural crops (i.e., field crops, legume vegetables, orchard/vineyards) via aircraft, chemigation, groundboom, airblast, mechanically pressurized handgun, and handheld and stationary fogger equipment. Applications at industrial, commercial, and residential sites can be made using handheld equipment such as low-pressure handwand sprayers, backpack sprayers, hose-end sprayers, handgun sprayers, ready-to-use aerosol cans, foggers, and trigger-pump sprayers. Labels vary with respect to requirements for Personal Protective Equipment (PPE) for occupational handlers. Those labels that do not specify any requirements for work attire have been assessed for residential handlers. Labels that require long-sleeve shirts, long pants, shoes, and socks (i.e., baseline attire), and chemical-resistant gloves have not been assessed for residential handlers. For applications using handheld foggers, in addition to baseline attire, workers are required to wear respiratory protection. Additionally, the labels for the registered uses covered under the Worker Protection Standard (WPS) (i.e., production of agricultural plants on a farm, forest, nursery, or greenhouse) stipulate a restricted

entry interval (REI) of 12 hours.

Humans may be exposed to PBO in food and drinking water based on its existing registered uses, since it may be applied directly to growing and post-harvest crops, and applications may result in PBO reaching surface and ground water sources of drinking water. Occupational inhalation handler and post-application exposures are expected. Inhalation and incidental oral ingestion exposures are expected for the residential scenarios. Spray drift exposures are also expected.

The chemical structure of PBO is shown below (Figure 1):

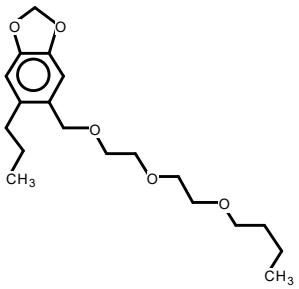

Figure 1. Structure of PBO

EVALUATION OF CARCINOGENICITY STUDIES

A. Carcinogenicity Study in Mice

Reference: Hermansky, S.J. and Wagner, C.L. (1993) Chronic dietary oncogenicity study with piperonyl butoxide in CD-1® mice. Bushy Run Research Center, Union Carbide Chemicals and Plastics Company, Inc., Export, PA. Laboratory Study ID: 91N0134, August 27, 1993. Piperonyl Butoxide Task Force II. Unpublished. MRID 42903701.

1. Experimental Design

In a carcinogenicity study (MRID 42903701), groups of 60 CD-1® mice/sex were administered PBO (90.78% a.i.; Lot # FEP-100 12/12/89) via the diet at dose levels of 0 (Group 1), 0 (Group 2), 30, 100, or 300 mg/kg/day for up to 78 weeks. No explanation was provided in the study report as to why two concurrent control groups were used. Evaluations (*i.e.*, body weight/body weight gain, food consumption, hematology, organ weight, and macroscopic/microscopic pathology) were conducted on all surviving animals during the study and/or at scheduled termination.

2. Survival Analyses

[The following text was extracted from the PBO qualitative risk assessment memo (L. Brunsman, TXR 00127670, 1/04/1995).]

The statistical evaluation of mortality indicated no significant incremental changes with increasing doses of PBO in female mice. Male mice showed a decreasing trend in mortality with increasing doses of PBO. See **Tables 1 and 2** for mouse mortality test results. The statistical evaluation of mortality was based upon the Thomas, Breslow, and Gart computer program.

Table 1. PBO - Charles River CD-1 Mouse Study
Male Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (mg/kg/day)	Weeks				Total
	1-26	27-52	53-68	69-79 [f]	
0[#]	2/120	5/118	13/113	20/100	40/120 (33) *[n]
30	1/60	2/59	6/57	10/51	19/60 (32)
100	0/60	3/60	3/57	10/54	16/60 (27)
300	0/60	0/60	7/60	6/53	13/60 (22)

[+]Number of animals that died during interval/Number of animals alive at the beginning of the interval.
Two separate control groups were combined for this analysis since there were no statistical differences in the tumor count or mortality of the two separate control groups for PBO.
[f]Final sacrifice at week 79.
[n]Negative trend.
( )Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.

Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 2. PBO - Charles River CD-1 Mouse Study
Female Mortality Rates[+] and Cox or Generalized K/W Test Results

Dose (mg/kg/day)	Weeks				Total
	1-26	27-52	53-65	66-80 [f]	
0[#]	2/120	2/118	6/116	20/110	30/120 (25)
30	1/60	1/59	4/58	10/54	16/60 (27)
100	0/60	0/60	8/60	15/52	23/60 (38)
300	2/60	1/58	5/57	12/52	20/60 (33)

[+]Number of animals that died during interval/Number of animals alive at the beginning of the interval.
[#]Two separate control groups were combined for this analysis since there were no statistical differences in the tumor count or mortality of the two separate control groups for PBO.
[f]Final sacrifice at week 79.
( )Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

3. Discussion of Tumor Data

HED Analyses
[The following text was extracted from the PBO qualitative risk assessment memo (L. Brunsman, TXR 0012767, 01/04/1995).]

Liver Tumors

Male mice had significant increasing trends in hepatocellular adenomas, carcinomas, and combined adenomas and/or carcinomas, all at $p<0.01$. There were significant differences in the pair-wise comparisons of the 100 mg/kg/day dose group with the controls for hepatocellular adenomas and combined adenomas and/or carcinomas, and significant pair-wise comparisons of the 300 mg/kg/day dose group with the controls for hepatocellular adenomas, carcinomas, and combined adenomas and/or carcinomas, all at $p<0.01$. The statistical analyses of male mice were based upon Peto's prevalence test since there was a statistically significant negative trend for mortality in male mice with increasing doses of PBO.

Female mice had a significant dose-related increasing trend, and a significant difference in the pair-wise comparison of the 300 mg/kg/day dose group with the controls for adenomas, both at $p<0.01$. There were no carcinomas reported in the females. The statistical analyses of female mice were based upon the Exact trend test and the Fisher's Exact test for pair-wise comparisons.

Tables 3 (males) and **4** (females) illustrate the tumor incidences and present a statistical evaluation of

these data.

Historical Controls

The in-life start date of the study is 1991, so historical control data for liver tumors between 1986-1996 (+/- 5 years from the in-life data) are preferable based on current Agency practice. For the historical control data presented below and in Tables 3 and 4, it was not possible to extract data from this specific range based on format in which the historical control data were presented.

Historical control data for liver tumors from the conducting laboratory (Bushy Run Research Center) are based on 8 studies from 59-70 mice of CD-1 strain. All studies were conducted over the years 1983-1990 (Summary of the Bushy Run Research Center (BRRC) Neoplasm Historical Control Database in CD-1 mice).

Liver adenomas: Males: mean= 13.6%, range= 8.7-21.7%
Females: mean = 0.8%, range = 0-1.7%

Liver carcinomas: Males: mean = 2.4%, range = 0-5%

The CARC also considered available Charles River Breeding Laboratory historical control summary data 1984-1991 (Refer to "Spontaneous neoplastic lesions in the Crl:CD-1 (ICR]BR mouse", March 1995, prepared by Patricia L. Lang, Ph.D.[1]).

The 18-month study data for liver tumors were taken from 12 study groups of 769-770 CD-1 mice from 1984-1991 (Tables A1 and A2, Lang, P., 1995).

Liver adenomas: Males: mean: 10.78%; range: 0-19.23%
Females: mean: 0.65%; range: 0-2%

Liver carcinomas: Males: mean: 4.94%; range: 1.25-11.54%

The 21-month study data for liver tumors were taken from 7 study groups of 318-370 CD-1 mice from 1985-1990 (Tables B1 and B2, Lang, P., 1995).

Adenomas: Males: mean: 7.57%; range: 0-12%
Females: mean: 1.26%; range: 0-2%

Carcinomas: Males: mean: 5.41%; range: 0-12%

The incidence for both male (all dose groups) and female adenomas (high dose group) and carcinomas for males (high dose group) were in excess of these historical control data.

[1] https://www.criver.com/sites/default/files/resources/doc_a/SpontaneousNeoplasticLesionsintheCrlCD-1%C2%AEBRMouse%E2%80%94March1995.pdf

Table 3. PBO – Male Hepatocellular Tumor Rates+ in Mice and Peto's Prevalence Test Results (*p*-values)

	Dose (mg/kg/day)					
	0#	30	100	300	Historical Controls[e] Mean % Range %	Historical Controls[f] Mean % (Range %)
Adenomas						
Incidence	17[a]/107	13/57	22/56	28/59	13.6%	10.78% (0-19.23%) 18-month studies
(%)	(16)	(23)	(39)	(47)	8.7-21.7%	7.57% (0-12%) 21-month studies
***p* =**	**0.000****	0.111	**0.001****	**0.000****		
Carcinomas						
Incidence	4/100	3/51	2/54	7[b]/53	2.4%	4.94% (1.25-11.54%) 18-month studies
(%)	(4)	(6)	(4)	(13)	0-5%	5.41% (0-12%) 21-month studies
***p* =**	**0.008****	0.260	0.499	**0.005****		
Combined (Carcinomas + Adenomas)						
Incidence	21/107	15[c]/57	24/56	30[d]/59	-	-
(%)	(20)	(26)	(43)	(51)		
***p* =**	**0.000****	0.116	**0.001****	**0.000****		

\+ Number of tumor-bearing animals/Number of animals examined,0 excluding those that died before observation of the first tumor.

\# Two separate control groups were combined for this risk assessment.

a First adenoma observed at week 61, dose 0 mg/kg/day.

b First carcinoma observed at week 69, dose 300 mg/kg/day.

c One animal in the 30 mg/kg/day dose group had both an adenoma and a carcinoma.

d Five animals in the 300 mg/kg/day dose group had both an adenoma and a carcinoma.

e Historical Control Data are based on 8 studies from 59-70 CD-1 strain of mice. All studies were from the Bushy Run Research Center and were conducted over the years 1983-1990.

f Historical control data are from Charles River Breeding Laboratory, March 1995. 18-month study data were taken from 12 studies from 1984-1991. 21-month study data were taken from 7 studies from 1985-1990.

Note: Significance of trend noted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then p<0.05. If **, then p<0.01

Table 4. PBO – Female Hepatocellular Tumor Rates[+] in Mice and Exact Trend Test and Fisher's Exact Test Results (*p*-values)

	Dose (mg/kg/day)					
	0[#]	30	100	300	Historical Controls[b] (1983-1990) Mean % Range %	Historical Controls[d] Mean% Range %
Adenomas[c]						
Incidence	4[a]/116	1/58	1/60	10/57	0.8%	0.65% (0-2%) 18-month studies
(%)	(3)	(2)	(2)	(18)	0-1.7%	1.26% (0-2%) 21-month studies
***p* =**	**0.000****	0.459[n]	0.444[n]	**0.003****		

\+ Number of tumor-bearing animals/Number of animals examined, excluding those that died before week 53.
\# Two separate control groups were combined for this analysis.
n Negative change from control.
a First adenoma observed at week 56, dose 0 mg/kg/day.
b Historical Control Data are based on 8 studies from 59-70 CD-1 strain of mice. All studies were from the Bushy Run Research Center and were conducted over the years 1983-1990.
c There were no hepatocellular carcinomas diagnosed.
d Historical control data are from Charles River Breeding Laboratory, March 1995. 18-month study data were taken from 12 studies from 1984-1991. 21-month study data were taken from 7 studies from 1985-1990.

Note: Significance of trend noted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then p<0.05. If **, then p<0.01

Registrant Submitted Data (MRID 51692501): As part of the new data package for the current evaluation, the registrant submitted a 1995 pathology re-evaluation of the mouse liver tumor data by Butler *et al.* 1995 (MRID 51692508). The peer review was not a formal review conducted according to the Pathology Working Group (PWG) guidance. To re-consider the original pathology reading, EPA typically requires a formal Pathology Work Group (PWG), not just a single peer review[2]. However, in this case, HED agreed to consider this information in their overall review of the tumors, but ultimately put more emphasis on the tumor incidences as reported in the original study. The registrant also submitted a statistical analysis of the tumor re-evaluation data in male mice (Lee, 2013, MRID 51692500, Attachment B) (See **Table 5a**). Following the initial assessment by pathologists at the conducting laboratory, re-evaluation of slides took place by Drs. W. Butler, W.R. Brown, W.W. Carlton, and R.A. Squire. The report states that a peer review was requested due to differences of opinion in the diagnosis of liver neoplasia commonly found among pathologists. "The consensus opinion was that males dosed at 100 and 300 mg/kg/day and females dosed at 300 mg/kg/day showed an increase in adenomas of the liver. The increased incidence of adenomas was in lesions of the eosinophilic type commonly associated with non-genotoxic mixed function oxidase inducers. Based on the re-read, the incidence of carcinomas was not statistically increased in any

[2] https://www.epa.gov/pesticide-registration/prn-94-5-requests-re-considerations-carcinogenicity-peer-review-decisions

treated group." The re-read of the tumors in the females was exactly the same as the original read, therefore, no additional statistics were performed by the registrant on these tumor data.

A trend test analysis for male mice indicated a significant increase (p <0.001) in the incidence of hepatocellular adenoma and in the combined incidence of hepatocellular adenoma and carcinoma, but not for the incidence of hepatocellular carcinoma (Lee, 2013, MRID 51692500, Attachment B). As stated by Lee 2013, the conclusions were essentially the same in the analyses based on the Fishers exact test (which does not take survival into account) and the Peto test (which does consider survival). See **Table 5a**.

Table 5a. Hepatic effect and liver tumor formation[+] in male and female CD-1 mice following treatment with PBO for 79 weeks (Excerpted from MRID 51692501)

Parameter	PBO in diet (mg/kg/day)[a]									
	Male mice					Female mice				
	0	0	30	100	300	0	0	30	100	300
Number of mice examined	60	60	60	60	60	60	60	60	60	60
Hepatocellular adenoma[+],[b, c]	8	7	13	21**	25***	2	2	1	1	10*
Hepatocellular carcinoma[+b,c]	3	3	2	2	5	0	0	0	0	0
Combined hepatocellular adenoma and carcinoma[b]	11	10	15	23**	30***	-	-	-	-	-

+ Number of tumor bearing animals.

[a] Groups of male and female mice were fed diets containing PBO to provide daily intakes of 0 (control), 30, 100 and 300 mg/kg/day. There were two male and female control groups in this study. Level of statistical significance: * p<0.05; **p<0.01; ***p<0.001.

[b] Data for liver tumor incidence in male mice taken from Butler (1995) with statistical analysis by Lee (2013). For male mice trend test analysis indicated a significant increase (p <0.001) in the incidence of hepatocellular adenoma and in the combined incidence of hepatocellular adenoma and carcinoma, but not for the incidence of hepatocellular carcinoma.

[c] Data for liver tumor incidence in female mice taken from USEPA (1995).

In consideration of this new data, HED conducted their own *ad hoc* analysis of the male mouse liver tumor data from the re-evaluation which resulted in findings that were similar to the registrant's statistics (**Table 5b**). The noted difference in the statistical analysis of the pathology re-read for the male mouse tumor data run by the registrant and HED's *ad hoc* analysis vs HED's 1995 statistical analysis is that there was no statistically significant trend or pair-wise comparison of any dosed group with controls for the carcinomas with the re-evaluation data, while the original statistics resulted in a significant trend and pairwise significance at the high dose for carcinomas. This difference was the result of fewer carcinomas being diagnosed in males in the dosed groups in the reevaluation. While the CARC ultimately put more emphasis on the original pathology results for male mice, it is noted that the statistical results for adenomas and the combined liver adenomas and/or carcinomas were similar for both evaluations. The resulting conclusion was the same--that the combined liver tumors, driven by adenomas, were treatment related in male mice at ≥100 mg/kg/day.

Table 5b: PBO – Male Charles River CD-1 Mouse Liver Tumor Rates+ and *ad hoc* Fisher's Exact Test Results Based on the 1995 Independent Re-Read of Dr. W. Butler (MRID 51692500) (*p*-values)				
	Dose (mg/kg/day)			
	0#	30	100	300
Adenomas				
Incidence	15/120	13/60	21/60	25/60
(%)	13	22	35	42
***p* =**	0.00001**	0.08532	0.00051**	0.00002**
Carcinomas				
Incidence	6/120	2/60	2/60	5/60
(%)	5	3	3	8
***p* =**	0.13566	0.81109	0.81109	0.28413
Combined (Carcinomas + Adenomas)				
Incidence	21/120	15/60	23/60	30/60
(%)	18	25	38	50
***p* =**	0.00001**	0.16139	0.00230**	0.00001**

+ Number of tumor-bearing animals/Number of animals examined.
# Two separate control groups were combined for this risk assessment.
Note: Significance of trend noted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then p<0.05. If **, then p<0.01

4. Non-neoplastic Lesions

Selected non-neoplastic microscopic pathology findings in mice are presented in **Table 6**. As stated in the study report, separate statistics were run for each control group. An increased incidence ($p<0.05$) of hepatocellular hypertrophy was observed in the 100 mg/kg/day males (16/60 treated vs. 6/60 Group 1 control only). At 300 mg/kg/day, treatment-related non-neoplastic microscopic findings included increased ($p\leq0.01$) incidences of hepatocellular hypertrophy in males (43/60 treated vs. 6-11/60 both controls) and females (9/60 treated vs. 0-4/60 Group 1 control), eosinophilic foci in males (5/60 treated vs 1/60 controls, not significant (NS)) and females (4/60 treated vs 0/60 controls, NS), and hemorrhage in males (13/60 treated vs. 1-2/60 controls) and females (7/60 (NS) vs 3/60 (both controls)). A slight increase in eosinophilic foci was observed at 100 mg/kg/day in females. There was also a slight increased incidence (NS) of hyperplasia at the high dose in both sexes.

The registrant reported that the hepatocyte hypertrophy was diffuse throughout the liver lobule in male mice given 300 mg/kg/day PBO but was mainly centrilobular in male and female mice given 100 mg/kg/day and in female mice given 300 mg/kg/day PBO, respectively.

Table 6. Selected non-neoplastic microscopic pathology findings in mice after administration of PBO in the diet for up to 78 weeks. [a]

Tissue	Dose (mg/kg/day)									
	Males					Females				
	0 (Group 1)	0 (Group 2)	30	100	300	0 (Group 1)	0 (Group 2)	30	100	300
# of animals examined	60	60	60	60	60	60	60	60	60	60
Liver										
Hepatocellular hypertrophy	6	11	11	16*	43** §§	0	4	0	1	9**
Eosinophilic foci[b]	1	0	0	0	5	0	0	0	1	4
Hepatocellular necrosis	21	20	11	14	29	6	13	13	17*	7
Hemorrhage	2	1	3	2	13** §§	3	3	1	4	7
Hepatocellular Hyperplasia	0	2	1	2	5	0	0	0	1	4

a Data were obtained from Appendix 3, Tables 15 and 19 on pages 194 and 241-242 of MRID 42903701.
b Taken from registrant MOA submission (MRID 51692501).
* Significantly different from Group 1 control; p≤0.05.
** Significantly different from Group 1 control; p≤0.01.
§§ Significantly different from Group 2 control; p≤0.01.

5. Adequacy of Dosing

The CARC concluded that dosing in the mouse carcinogenicity study was adequate and not excessive to assess carcinogenicity. This was based on liver histopathology (eosinophilic foci and hyperplasia) seen at 300 mg/kg/day in males and at ≥100 mg/kg/day in females. Liver hypertrophy was also seen in males at ≥100 mg/kg/day and in females at 300 mg/kg/day. Increased liver weights were seen in both sexes at ≥100 mg/kg/day. Survival was not adversly affected.

The CARC determined that the combined hepatocellular adenomas and/or carcinomas, driven by adenomas, seen at ≥100 mg/kg/day in male mice are treatment related at doses that were considered to be adequate, and not excessive, for evaluating carcinogenicity. *This was based on significant increasing trends in hepatocellular adenomas and combined adenomas and/or carcinomas, as well as significant differences in the pair-wise comparisons of the 100 and 300 mg/kg/day dose groups with the controls for hepatocellular adenomas and combined adenomas and/or carcinomas, all at p<0.01. For carcinomas, there was a significant increasing trend and significant difference in the pair-wise comparison of the 300 mg/kg/day dose group, both at p<0.01. The incidences of liver adenomas at 100 and 300 mg/kg/day were outside the historical control ranges of both the conducting laboratory and Charles River Laboratories. The incidence of liver carcinomas at 300 mg/kg/day was just outside the historical control ranges from both the conducting laboratory and Charles River Laboratories. Supporting pre-neoplastic lesions (liver hyperplasia and eosinophilic foci) were seen at 300 mg/kg/day at study termination. While the CARC relied primarily on the tumor data as reported in MRID 42903701, it also considered a 1995 pathology re-evaluation of the liver tumors. The overall conclusion, however, was the same--that the combined liver tumors, driven by adenomas, were treatment related.*

The CARC determined that the liver adenomas seen at 300 mg/kg/day in female mice were treatment related at a dose that was considered to be adequate, and not excessive, for evaluating carcinogenicity. *Female mice had a significant increasing trend, and a significant difference in the pair-*

wise comparison of the 300 mg/kg/day dose group with the controls, for adenomas, both at p<0.01. There were no carcinomas reported in the females. The incidences of liver adenomas at 300 mg/kg/day were outside both the conducting laboratory, Bushy Run Research Center and Charles River historical control ranges. Supporting pre-neoplastic lesions included liver hyperplasia and foci, which were seen at 300 mg/kg/day at the termination of the study.

6. Additional Carcinogenicity Studies in Mice

Several additional studies in mice, including those from the literature and the National Cancer Institute (NCI) which were considered at the 1995 CPRC meeting, or which were identified in a literature search for the current evaluation, are listed below.

- Takahashi, O., Oishi, S., Fujitani,T. *et al.* (1994a) Piperonyl butoxide induces hepatocellular carcinoma in male CD-1 mice. Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo, Japan. Study ID: Not provided. *Arch. Toxicol.* 68, pp. 467-469. MRID 52376006.

- Takahashi, O., Oishi, S., Fujitani,T. *et al.* (1997) Chronic toxicity studies of piperonyl butoxide in CD-1 mice: Induction of hepatocellular carcinoma. Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo, Japan. Study ID: Not provided. Toxicol. 124, pp. 95 103. MRID 52376009.

- National Cancer Institute (NCI, 1979) Bioassay of piperonyl butoxide for possible carcinogenicity. Frederick Cancer Research Center, Frederick, MD. NCI ID: NCI-CG-TR-120. National Institutes of Health (NIH) Publication No. 79-1375. MRID 52376001.

For the current meeting, these studies were evaluated according to the Agency's *Guidance for Considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment (8/28/2012, Office of Pesticide Programs, US EPA)* and Data Evaluation Records (DERs) were prepared. The NCI study [MRID 52376001] was determined to be Unacceptable; therefore, it was not included in the weight of evidence evaluation of carcinogenicity of PBO. Details are provided in the DER (J. Kidwell, TXR 0058676, 06/28/2024) regarding the Unacceptable status. The Takahashi *et al.* 1994 (MRID 52376006) and 1997 (MRID 52376009) studies in mice were determined to be Acceptable/Non-guideline and useful qualitatively to inform the liver tumor profile in mice for PBO. Details are provided in the DER (J. Kidwell, TXR 0058676, 06/28/2024) regarding the studies' acceptability status.

In the literature studies by Takahashi *et al*. 1994 (males only) and 1997 (both sexes) (MRIDs 52376006 and 52376009, respectively), male and female CD-1 mice dosed in the diet at doses of 0, 0.6% or 1.2% PBO (94.3% a.i.) (corresponding to 0, 816/876, 1692/2004 mg/kg/day) for 52 weeks showed liver tumors (adenomas + carcinomas) in male mice at the lowest dose tested of 816 mg/kg/day. No liver tumors were seen in female mice at the lowest dose tested of 876 mg/kg/day. It is noted that the first adenoma in the guideline mouse study (MRID 42903701) wasn't observed until week 56 in females and week 61 in males, and the first carcinoma in the guideline study wasn't observed until week 69 in males. In the Takahashi studies, liver tumors were observed in both sexes at a very high dose of 1692/2004 mg/kg/day which are 1.6-2X the limit dose of 1000 mg/kg/day. This dose was considered to

be excessive in both sexes based on the early mortality observed in females, the sustained >10% decreases in body weight in both sexes at 52 weeks (↓29% males, ↓19% females at termination), non-neoplastic lesions in the kidney (black colored) in males and ovaries (edema and hemorrhage), massive hemorrhages in the pleural cavity or gastrointestinal tract, without evidence of tumors, and massive hemorrhages in the peritoneal cavity due to liver cancers, observed in some 1692/2004 mg/kg/day animals that died prior to scheduled necropsy. In addition, studies conducted at greater than the limit dose are of limited use for regulatory purposes. It was concluded that this study supports that, following a chronic exposure to PBO, males are more susceptible to tumor formation than females.

B. Chronic Toxicity/Carcinogenicity Study in Rats

Reference: *Graham, C. 24-Month Dietary Toxicity Study and Carcinogenicity Study of Piperonyl Butoxide in the Albino Rat. Bio-Research Ltd., 87 Senneville Road, Senneville, Quebec H9X 3R3 Canada. Study No. 81690. Sponsor: Piperonyl Butoxide Task Force. August 12, 1987. MRID 40323701*

1. Experimental Design

Sprague-Dawley Crl-CDR strain rats (60/sex/dose group) were dosed as control-1, control-2, 30, 100, or 500 mg/kg/day by the dietary route for 24 months. No explanation was provided in the study report as to why two concurrent control groups were used. Additional groups of 10 animals/sex were included for the 0 (Group 2), 15, and 30 mg/kg/day groups and then these animals were euthanized after treatment for four weeks and macroscopic/microscopic liver evaluations were conducted; the 15 mg/kg/day animals were discontinued due to lack of liver findings.

2. Survival Analysis

[The following text was extracted from the PBO qualitative risk assessment memo (L. Brunsman, TXR 0012767, 01/04/1995).]

The statistical evaluation of mortality indicated no significant incremental changes with increasing doses (trend or pair-wise) of PBO in male rats. Female rats had a significant pair-wise comparison (negative) of the 100 mg/kg/day group compared to controls. See **Tables 7 and 8** for rat mortality test results. The statistical evaluation of mortality was based upon the Thomas, Breslow, and Gart computer program.

Survival rates up to scheduled euthanasia at Weeks 105 (males) and 106 (females) in the 0, 0, 30, 100, and 500 mg/kg/day groups were 18%, 22%, 13%, 18%, and 22% in males and 45%, 32%, 37%, 57%, and 50% in females, respectively. Unscheduled euthanasia in the 0, 0, 30, 100, and 500 mg/kg/day groups accounted for the deaths of 49, 47, 52, 49, and 47 males and 33, 41, 38, 26, and 30 females, respectively. Over the first 18 months, the mean survival rates were 75% for males and 87% for females, although mortality was increased in males during the last 6-7 months of treatment. Survival in males did not fall below 25% (lower limit recommended by OPPTS 870.4300 guideline) until Weeks 97-100; survival in females was greater than 25% in all groups up to termination. Ultimately, the males lived nearly a full lifespan and this mortality did not impact the tumor analyses.

Table 7. PBO- Charles River Sprague Dawley Crl-CDR Rat Study Male Mortality Rates[+] and Cox or Generalized K/W Test Results							
Dose (mg/kg/day)	Weeks						Total
	1-4	5	5-26	27-52	53-78	79-105 [f]	
0[#]	2/131	10/129	0/119	4/119	27/115 (23)	64/88 (73)	97/121 (80)
30	1/70	10/69	0/59	1/59	20/58 (38)	30/38 (79)	52/60 (87)
100	1/61	0/60	2/60	0/58	15/58 (26)	32/43 (74)	50/61 (82)
500	5/60	0/55	0/55	2/55	10/53 (19)	30/43 (70)	47/60 (78)

[+] Number of animals that died during interval/Number of animals alive at the beginning of the interval.
[#] Two separate control groups were combined for this risk assessment.
[f] Final sacrifice at week 105.
( )Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 8. PBO - Charles River Sprague Dawley Crl-CDR Rat Study Female Mortality Rates[+] and Cox or Generalized K/W Test Results							
Dose (mg/kg/day)	Weeks						Total
	1-4	5	5-26	27-52	53-78	79-107 [f]	
0[#]	1/131	10/130	2/120	2/118	17/116	53/99	75/121 (62)
30	2/72	10/70	1/60	2/59	6/57	29/51	40/62 (65)
100	1/61	0/60	0/60	1/60	6/59	19/53	27/61 (44)*[n]
500	0/60	0/60	1/60	1/59	7/58	21/51	30/60 (50)

[+] Number of animals that died during interval/Number of animals alive at the beginning of the interval.
[#] Two separate control groups were combined for this risk assessment.
[f] Final sacrifice at week 105.
[n] Negative pair-wise comparison.
( )Percent.
Note: Time intervals were selected for display purposes only.
Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

3. Discussion of Tumor Data

[The following text was extracted from the PBO qualitative risk assessment memo (L. Brunsman, TXR 0012767, 01/04/1995).]

Tables 9 and 10 below illustrate the tumor incidence and present a statistical assessment of the thyroid data.

Thyroid Tumors

Male rats had a significant increasing trend in thyroid follicular cell combined adenomas and/or carcinomas at $p < 0.05$. There were no significant differences in the pair-wise comparisons of the dosed groups with the controls.

Female rats had a significant increasing trend in thyroid follicular cell adenomas at $p < 0.05$. There were no significant differences in the pair-wise comparisons of the dosed groups with the controls.

The statistical analyses in male and female rats were based upon the Exact Trend Test and the Fisher's Exact test for pair-wise comparisons.

Historical Control Data:
The in-life start date for the chronic toxicity/carcinogenicity study in rats is 1984. Historical control data ±5 years from this date (1979-1989) are appropriate for comparison based on current Agency practice.

According to the study report (Appendix A, Vol 1, p. 61/380 of MRID 40323701), mean incidences (range) of combined thyroid follicular cell adenomas and/or adenocarcinomas from the conducting laboratory, Bio-Research Lab, historical data for Sprague Dawley rats from 1980-1986 are as follows:

> Males (565 animals examined; # tumors=16): Thyroid follicular cell adenomas/adenocarcinomas combined: 2.8% (0-7%).
>
> Females (565 animals examined; # tumors n=6): Thyroid follicular cell adenomas/adenocarcinomas combined: 1.06% (0-5%).

The Charles River Breeder's background summary (refer to "Spontaneous Neoplastic Lesions and Selected Non-neoplastic Lesions in the Crl:CD®BR Rat, Patricia Lang, February, 1992[3]) indicates a range of 1.1 to 25.7% for thyroid follicular adenomas and 1.0 to 6.0% for carcinomas for males and 1-14.5% adenomas for females. These data were taken from 19 control groups from 1984-1989.

[3] https://www.criver.com/sites/default/files/resources/doc_a/rm_rm_r_lesions_selected_non-neo_crlcdbr_rat.pdf

Table 9: PBO – Male Charles River Sprague Dawley Crl-CDR Rat Thyroid Follicular Cell Tumor Rates[+] and Exact Trend Test and Fisher's Exact Test Results

	Dose (mg/kg/day)					
	0[#]	30	100	500	Historical Controls[c] Mean % Range %	Historical Controls[d] Range %
Adenomas						
Incidence	2/115	1/58	0/58	2[a]/53		
(%)	2	2	0	4		1.1-25.7%[d]
***p* =**	0.212	0.740	0.441[n]	0.375		
Carcinomas						
Incidence	1[b]/115	0/58	0/58	2/53		
(%)	1	0	0	4		1-6%[d]
***p* =**	0.090	0.665[n]	0.665[n]	0.234		
Combined (Carcinomas + Adenomas)						
Incidence	3/115	1/58	0/58	4/53	2.8%[c]	
(%)	3	2	0	8	0-7%	
***p* =**	**0.040***	0.589[n]	0.291[n]	0.142		

\+ Number of tumor-bearing animals/Number of animals examined, excluding those that died or were sacrificed before week 53.

\# Two separate control groups were combined for this risk assessment.

n Negative change from control.

a First adenoma observed at week 79, dose 500 mg/kg/day.

b First carcinoma observed at week 89, dose 0 mg/kg/day.

c Bio-Research Lab Historical Control Data (Appendix A, Vol 1, p. 61/380) of MRID 40323701.

d Charles River Historical control data, Lang, P. Feb 1992 https://www.criver.com/sites/default/files/resources/doc_a/rm_rm_r_lesions_selected_non-neo_crlcdbr_rat.pdf

Note: Significance of trend noted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then p<0.05. If **, then p<0.01.

Table 10: PBO – Female Charles River Sprague Dawley Crl-CDR Rat Thyroid Follicular Cell Tumor Rates[+] and Exact Trend Test and Fisher's Exact Test Results (*p*-values)						
	Dose (mg/kg/day)					
	0[#]	30	100	500	Historical Controls[b] Mean % Range %	Historical Controls[c]
Adenomas						
Incidence	1/116	0/57	1[a]/58	3/58	1.06%	
(%)	1	0	2	5	0-5%	1-14.5%
***p* =**	**0.029***	0.671[n]	0.557	0.109		

\+ Number of tumor-bearing animals/Number of animals examined, excluding those that died or were sacrificed before week 53.

\# Two separate control groups were combined for this risk assessment.

n Negative change from control

a First adenoma observed at week 105, dose 100mg/kg/day.

b Bio-Research Lab Historical Control Data (Appendix A, Vol 1, p. 61/380)

c Charles River Historical control data, Lang, P. Feb 1992 https://www.criver.com/sites/default/files/resources/doc_a/rm_rm_r_lesions_selected_non-neo_crlcdbr_rat.pdf

Note: Significance of trend noted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then p<0.05. If **, then p<0.01.

Liver Tumors

Ad hoc statistics were performed on the liver tumors seen in male and female rats to corroborate the conclusions in 1995 CPRC report that the liver tumors were not treatment related due to the very slight numerical increase in tumors. Male rats had significant increasing trends in adenomas at p<0.01 and combined adenomas and/or carcinomas at p<0.05 (**Table 11a)**. There were no significant differences in the pair-wise comparisons for adenomas, carcinomas, or combined adenomas and/or carcinomas compared to controls. Female rats had no significant increasing trend or pair-wise comparison of any dosed group with the control for carcinomas (**Table 11b)**.

Historical control data: According to the study report (Appendix A, Vol 1, p. 61/380 of MRID 40323701), mean incidences (range) of liver neoplasms from the Bio-Research Lab historical data from 1979-1986 from 7 studies are as follows:

Males: adenomas 1.2% (0-3.3%), carcinomas 2.3% (0-5%), combined mean 4.25%
Females: adenomas: 0.7% (0-3%), carcinomas 0.2% (0-1.5%), combined mean 1.24%

Historical control data provided for other laboratories (in Appendix B, Vol 1, p. 64/380 or Appendix 55 Vol 9 of study report of MRID 40323701) reports historical control data for Charles River CD rats for in-life completion dates ranging from 1979-1985. The historical control mean (range) was listed as 0.8% (0-3.5%) for liver adenomas and 2.2% (0-11.1%) for liver carcinomas for males and 0.2% (0-1.3%) for adenomas and 1.7% (0-14.4%) for carcinomas for females.

Table 11a: PBO – Male Charles River Sprague Dawley Crl-CDR Rat Liver Tumor Rates[+] and *ad hoc* Exact Test Results						
	Dose (mg/kg/day)					
	0[#]	30	100	500	Historical Controls[a] Mean % Range %	Historical Controls[b] Range %
Adenomas						
Incidence	0/120	0/60	0/60	2/60	1.2%	0.8%
(%)	0	0	0	3	0-3.3%	0-3.5%
***p* =**	0.00269**	1.00000	1.00000	0.10987		
Carcinomas						
Incidence	2/120	0/60	0/60	1/60	2.3%	2.2%
(%)	2	0	0	2	0-5%	0-11.1%
***p* =**	0.35569	1.00000	1.0000	0.70619		
Combined (Carcinomas + Adenomas)						
Incidence	2/120	0/60	0/60	3/60	4.25%	
(%)	2	0	0	5		
***p* =**	0.01979*	1.0000	1.0000	0.20708		

Data were obtained from Volume 1, Pathologist's Report, page 52/380, of MRID 40323701.
Ad hoc stats performed by L. Brunsman, 04/17/2024.

+ Number of tumor-bearing animals/Number of animals examined.

Two separate control groups were combined for this risk assessment.

a Bio-Research Lab Historical Control Data (Appendix A, Vol 1, p. 61/380) (1979-1986) of MRID 40323701).

b Charles River Historical control data, (Appendix B, Vol 1, p. 64/380 or Appendix 55, Vol 9) (1979-1985) of MRID 40323701).

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 11b: PBO – Female Charles River Sprague Dawley Crl-CDR Rat Liver Tumor Rates+ and *ad hoc* Exact Test Results						
	Dose (mg/kg/day)					
	0#	30	100	500	Historical Controls[a] Mean % Range %	Historical Controls[b] Range %
Carcinomas						
Incidence	0/120	0/60	1/60	1/60	0.2%	1.7%
(%)	0	0	2	2	0-1.5%	0-14.4%
***p* =**	0.09784	1.00000	0.33333	0.33333		

Data were obtained from Volume 1, Pathologist's Report, page 52/380, of MRID 40323701.
Ad hoc stats performed by L. Brunsman, 04/17/2024.
+ Number of tumor-bearing animals/Number of animals examined.
# Two separate control groups were combined for this risk assessment.
Bio-Research Lab Historical Control Data (Appendix A, Vol 1, p. 61/380) (1979-1986) of MRID 40323701).
7 24-month studies, totalling 565 animals/sex.
[b] Charles River Historical control data, (Appendix B, Vol 1, p. 64/380 or Appendix 55, Vol 9) (1979-1985) of MRID 40323701). 24 month study. 880 animals/sex. Total number of studies not presented.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

4. Non-neoplastic Lesions

In the liver, increased ($p<0.05$) incidences of hepatocellular hypertrophy were observed relative to the Group 1 and 2 controls, respectively, in the 500 mg/kg/day males (29/60 treated vs. 4/60 1 and 2/60 controls) and females (47/60 treated vs. 4/60 and 2/60 controls), accompanied by slight non-dose-related increases (NS) in focal hepatocellular hyperplasia. A dose-related increase (NS) in eosinophilic focal cells was observed in males at ≥100 mg/kg/day. Focal mixed cells in females were increased ($p<0.05$) at ≥100 mg/kg/day (13/60 and 20/60 treated vs. 3/60 and 3/60 controls) (**Table 12**).

In the thyroid, increased ($p<0.05$) incidences of pigment accumulation in the follicles of the 500 mg/kg/day males (48/60 treated vs. 24/60 and 27/60 controls) and females (44/60 treated vs. 8/60 and 10/60 controls) were observed, accompanied by increases ($p<0.05$) in thyroid follicular cell hyperplasia in the 30 and 500 mg/kg/day males (13/60 and 21/60 treated vs. 4/60 and 11/60 controls) and the ≥100 mg/kg/day females (9-11/60 treated vs. 0/60 and 4/60 controls) (**Table 12**).

Table 12. Selected non-neoplastic microscopic pathology findings in rats administered PBO in the diet for up to 104/105 weeks (males/females). [a]

Parameter	Concentration (mg/kg/day)				
	0 (Grp 1)	0 (Grp 2)	30	100	500
Males					
Liver (Tissues examined)	**60**	**60**	**60**	**60**	**60**
Hepatocyte hypertrophy	4	2	1	4	29*, §
Hepatocyte hyperplasia	1	1	1	5	4
Focal mixed cells	1	1	4	1	5
Focal eosinophilic cells	6	6	3	10	12
Focal basophilic cells	11	6	5	4	5
Focal clear cells	2	3	2	3	1
Thyroid glands (Tissues examined)	**60**	**59**	**60**	**60**	**60**
Pigment in follicles	24	27	22	22	48*, §
Follicular cell hypertrophy	0	1	1	2	1
Follicular cell hyperplasia	4	11	13*	8	21*, §
Parafollicular cell hyperplasia	19	14	4*, §	12	7*
Females					
Liver (Tissues examined)	**60**	**60**	**60**	**60**	**60**
Hepatocyte hypertrophy	4	2	0	2	47*, §
Hepatocyte hyperplasia	5	5	5	12	8
Focal mixed cells	3	3	3	13*, §	20*, §
Focal eosinophilic cells	6	2	5	5	3
Focal basophilic cells	13	7	3*	8	3*
Focal clear cells	2	2	3	2	0
Thyroid glands (Tissues examined)	**60**	**60**	**60**	**59**	**60**
Pigment in follicles	8	10	6	9	44*, §
Follicular cell hypertrophy	0	2	1	0	3
Follicular cell hyperplasia	0	4	2	9*	11*, §
Parafollicular cell hyperplasia	13	14	14	9	9

a Data were obtained from pages 53-58, 68-69, and 73-75 (Vol. 1) of MRID 40323701.
* Significantly different from Group 1 control; p<0.05.
** Significantly different from Group 1 control; p<0.01 (Chi-square).
§ Significantly different from Group 2 control; p<0.05.

5. Adequacy of Dosing

Dosing was considered adequate, and not excessive, to assess the carcinogenic potential of PBO. This was based on liver effects, including increased liver weights in males at 500 mg/kg/day and in females at ≥30 mg/kg/day, macroscopic findings (enlarged and pale liver) and hepatocyte hypertrophy at 500 mg/kg/day (both sexes), and focal mixed cells in the liver at ≥100 mg/kg/day in females. Thyroid follicular cell hyperplasia was observed at ≥100 mg/kg/day in females. Decreased body weights of 11-21% were seen at 500 mg/kg/day in both sexes. Total cholesterol was increased in both sexes at ≥100 mg/kg/day. No statistically significant increases in mortality were seen for either sex compared to controls. The mortality in males observed at the very end of the study did not compromise the integrity of the study overall and still allowed for valid statistical comparisons of tumors.

The CARC concluded that the combined thyroid follicular cell adenomas and/or carcinomas seen in male rats and the thyroid follicular cell adenomas seen in female rats are not treatment related at doses that were considered to be adequate, and not excessive, for evaluating carcinogenicity. *For*

males, this was based on a significant increasing trend only in thyroid follicular cell combined adenomas and/or carcinomas at p < 0.05, but no significant differences seen in the pair-wise comparisons of any dosed group with the controls for thyroid follicular cell adenomas, carcinomas or combined. In addition, there was a lack of a dose response at the lower doses. The incidences of thyroid follicular cell adenomas or carcinomas alone at 500 mg/kg/day were within the Charles River historical control ranges. The incidences of thyroid follicular cell combined adenomas and/or carcinomas at 500 mg/kg/day are just above the conducting laboratory historical control range. For females, this was based on a significant increasing trend only in thyroid follicular cell adenomas at p<0.05, but no significant differences in the pair-wise comparisons of the dosed groups with the controls. There were no thyroid follicular cell carcinomas reported in the female rats. The incidences of adenomas were within the conducting laboratory and Charles River Laboratories historical control ranges.

The CARC concluded that the liver tumors seen in male and female rats were <u>not</u> treatment related. *For males, this was based on significant increasing trends only in adenomas at p<0.01 and combined adenomas and/or carcinomas at p<0.05, but no significant differences in the pair-wise comparisons of adenomas, carcinomas or combined compared to controls at any dose. For females, this was based on no significant trend or pair-wise comparisons of any dosed group with the controls for carcinomas. There were no liver adenomas reported for females. For both sexes, the incidences of liver adenomas and/or carcinomas were within or just outside the in-house and Charles River historical control ranges.*

<u>6. Additional Carcinogenicity Studies in Rats</u>

Several additional studies in rats, including those from the literature and the NCI, were considered at the 1995 CPRC meeting or were identified in a literature search for the current meeting are listed below.

- Takahashi et al. 1994b. Chronic Toxicity Studies of Piperonyl Butoxide in F344 Rats: Induction of Hepatocellular Carcinoma. *Fundamental and Applied Toxicology* 22, 293-303. MRID 52376010.

- National Cancer Institute (NCI; 1979) Bioassay of piperonyl butoxide for possible carcinogenicity. Frederick Cancer Research Center, Frederick, MD. NCI ID: NCI-CG-TR-120. National Institutes of Health (NIH) Publication No. 79-1375. MRID 52376001.

- Maekawa, A., Onodera, H., Furuta, K., *et al.* (1985) Lack of evidence of carcinogenicity of technical grade piperonyl butoxide in F344 rats: Selective induction of ileocecal ulcers. Division of Pathology, National Institute of Hygienic Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan. Study ID: Not provided. *Fd. Chem. Toxic.* Vol. 23, No. 7, pp. 675-682. MRID 52376002.

For the current assessment, these studies were evaluated according to the Agency's *Guidance for Considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment (8/28/2012, Office of Pesticide Programs, US EPA)* and DERs were prepared. The NCI study was determined to be Unacceptable; therefore, it was not included in the weight of evidence evaluation of carcinogenicity of PBO. Details are provided in J. Kidwell, TXR 0058676, 06/28/2024 for MRID 52376001 regarding the Unacceptable status. The Takahashi *et al* 1994b and Maekawa *et al.* 1985 studies were determined to be Acceptable/Non-guideline, for qualitative use only, in support of the tumor profile for PBO. Details are provided in J. Kidwell, TXR 0058676, 06/28/2024 for MRIDs

52376010 (Takahashi *et al.* 1994b) and 52376002 (Maekawa *et al.* 1985).

Limited qualitative support for the lack of tumors in Sprague Dawley rats seen at 500 mg/kg/day in the guideline study (MRID 40323701) was found in literature studies by Takahashi *et al*. 1994 (MRID 53276010) and Maekawa *et al*. 1985 (MRID 52376002), which were both conducted in Fischer rats. In the Takahashi *et al.* study, Fischer rats that were given dietary doses of 0, 0.6, 1.2 and 2.4% PBO (≥94.3% a.i) (equivalent to 0, 547/537, 1052/1061, 1877/2002 mg/kg/day in males/females, respectively) in the diet for 96 weeks showed no liver tumors at the lowest dose tested of 547/537 mg/kg/day in both sexes. There were liver tumors seen at the top two doses (both of which were ≥ limit dose of 1000 mg/kg/day as recommended by the OPPTS 870.4300 guideline), however, both of these doses were also considered to be excessive, based mainly on excessive decreases in body weights, large increases in liver weights, anemia, severe clinical chemistry changes, increased mortality due to cecal hemorrhage, and black colored kidneys. Therefore, any tumors seen at these doses were not considered relevant for the evaluation of a tumor response. In the Maekawa *et al*. 1985 study, Fischer rats that were dosed with PBO (89% a.i) in the diet at concentrations of 0, 0.5, or 1.0% (roughly equivalent to 0, 250 and 500 mg/kg/day of PBO) for up to 104 weeks demonstrated no carcinogenic activity.

IV. TOXICOLOGY

A. Metabolism

In a metabolism study (MRID 45582701), a mixture of non-radiolabeled (93.4 % a.i.; Lot/Batch No. SF-97-004) and phenyl labeled ^{14}C-PBO (100% radiochemical purity; Lot/Batch No. 980319/RP2) was administered to 4 CRL:CD rats/sex/dose by single gavage exposure at dose levels of 50 or 500 mg/kg body weight. The main route of excretion was via feces which contained 82.9-85.1% of the administered radioactivity at the low dose level and 64.1-75.9% at the high dose level at 168 hours. The percent radioactive dose excreted in the urine at 168 hours was 11.1-14.4% in the low dose group and 19.5-30.2% in the high dose group. The majority of the administered radioactivity was excreted in 0-48 hours urine and feces samples in both dose groups. The percent of administered dose in the carcass was below 0.5% in the low dose and high dose groups. The total percent of radioactive dose recovered in both dose groups ranged between 97.4% and 99.6%. There was no significant difference in the excretion pattern between the two dose groups or between sexes in the same dose group.

Two major metabolites were excreted in feces. The first was identified as unchanged PBO, corresponding to 15.6-23.9% of the administered dose. The second was identified as PBO with methylenedioxy ring opened to form catechol and found at 17.4-19.7% of the administered dose. Two other minor metabolites were also identified but were present in low amounts (4-6% of administered radioactivity) in the high dose group.

Several radioactive peaks (~20 peaks) were observed in urine samples, and none of these individual peaks exceeded 5% of the administered radioactivity. Metabolites in urine occurred at a maximum of 3% of the administered dose in males and 9% in females. While there was no significant difference in the excretion profile between sexes, four metabolites were found primarily in the urine of females, while one other was found only in the urine of males.

Based on the identification of metabolites, there are three major reactions in the metabolism of PBO: 1) Opening of the methylenedioxy ring to form the catechol; 2) Sequential cleavage of the 2-(2-butoxyethoxy)ethoxymethyl side chain to produce a series of alcohols and acids; 3) Conjugation of one of the phenolic groups to yield a glucuronide, sulphate, or methoxy derivative.

There are no metabolism data for the mouse.

B. Mutagenicity

The acceptable mutagenicity studies satisfy the three categories of mutagenicity testing (pre-1991 guidelines) of gene mutations, structural chromosomal aberrations, and other mechanisms of genotoxic effects. In summary, PBO did not cause genetic damage when tested in a series of *in vitro* assays including bacterial and mammalian gene mutations, chromosomal aberration tests (Chinese hamster ovary (CHO) cells), a sister chromatid exchange assay (CHO cells), and an unscheduled DNA synthesis assay (rat primary hepatocytes and human liver slices). Overall, the weight of evidence does not suggest that PBO has genotoxic potential.

Table 13 presents the individual mutagenicity studies that are considered to be acceptable. Two additional literature studies (Butler *et al*. 1996 (MRID 52376007) and Beamand *et al*. 1996 (MRID 52376005)) were identified in a literature search and included in the overall WOE for mutagenicity.

Table 13. Mutation/Genotoxicity of PBO

Guideline No. Study Type	MRID No. (year) Classification	Results/Dose Levels
870.5100 Bacterial Reverse Mutation Assay	42004502 (1991) **Acceptable/Guideline** TXR 0009789	No evidence of mutagenicity in *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 or TA100 in the presence or absence of metabolic activation (no cytotoxicity noted) Doses 100-5000 µg/plate
870.5100 Bacterial Reverse Mutation Assay NTP	00143499 (1983) **Acceptable/Guideline** TXR No. 0007674	No evidence of gene mutations in *S. typhimurium* (TA 1535, TA1537, TA98, TA100) with and without metabolic activation. Dose up to 10 mg/plate
870.5300 *In vitro* Mammalian Cell Gene Mutation Assay	00147693 (1985) **Acceptable/Guideline** TXR 0004508	In the presence of S-9, PBO produced no increase in mutants at concentrations up to 500 µg/ml (44% relative toxicity). Equivocal response in 75 µg/ml only under non-activated conditions in CHO cells. Doses 10-100 µg/ml (without activation) Doses 25-500 µg/ml (with activation)
870.5375 *In vitro* Mammalian Cell Chromosomal Aberrations Test	43013801 (1993) **Acceptable/Guideline** TXR 0011005	No induction of chromosomal aberration in CHO cells Doses in non-activated condition: 15.0-30.0 µg/mL for 10 or 20 hours Doses in S9 activated condition: 12-120 µg/mL for 2, 10, 20 or 30 hours
870.5375 *In vitro* Mammalian Cell Chromosomal Aberrations Test	00143499 (1983) **Acceptable/Guideline** TXR 0007674	No evidence of induction of chromosomal aberrations in CHO cells

Table 13. Mutation/Genotoxicity of PBO		
Guideline No. Study Type	**MRID No. (year) Classification**	**Results/Dose Levels**
NTP		
870.5550 Unscheduled DNA Synthesis in Rat Primary Hepatocytes	42004503 (1991) **Acceptable/Guideline** TXR 0009789	No evidence of induction of unscheduled DNA synthesis Dose Levels tested: 5,10, 25 and 50 µg/ml Cytotoxic at ≥74.9 µg/ml
Non-guideline *In vitro* genotoxicity studies	MRID 52376007 (1996) WH Butler, KL Gabriel, FJ Preiss TG, Osimitz, 1996. Lack of genotoxicity of piperonyl butoxide. Mutation Research 371 (1996) 249-258 **Acceptable/Non-Guideline** TXR 0058676	*Reverse Mutation Assay*: No evidence of mutagenicity in *S. typhimurium* strains TA1535, TA1537, TA1538, TA98 or TA100 in the presence or absence of metabolic activation Doses 100-5000 µg/plate (+/−S9). *Chromosomal Aberration Assay using CHO cells*: No increases in the percentage of cells with aberrations were observed at any concentration after exposure for 10 or 20 hours with or without metabolic activation. Doses: 0, 25.0, or 49.9 µg/mL (−S9) and 0, 62.6, 125, 188, or 251 µg/mL (+S9) for 7.25 hours and fixed at 10 hours after initiation or 0, 49.9, 74.9, or 99.9 µg/mL (−S9) and 62.6, 125, 188, or 251 µg/mL (+S9) for 17.25 hours. *HGPRT locus gene mutation assay*: The cloning efficiency of CHO cells demonstrated that PBO was cytotoxic at 100 µg/mL (+S9) and at 500 µg/mL (−S9). In the absence of metabolic activation, mutation frequency was increased only at 75 µg/mL, with a slight dose-dependent increase in mutation. In the presence of metabolic activation, there was no evidence of a dose-dependent increase in mutation. Overall, there was no evidence of induced mutant colonies over background in the presence or absence of S9 activation. *Unscheduled DNA Synthesis experiment*: Treatment with PBO did not induce changes in nuclear labeling of the primary hepatocytes at any tested concentration up to 49.9 µg/mL. Hepatocytes at concentrations of 2.5-49.9 µg/mL.
Non-Guideline Genotoxicity Unscheduled UDS Synthesis	MRID 52376005 (1996) Beamand J *et al.* 1996. Lack of effect of piperonyl butoxide on unscheduled DNA synthesis in precision-cut human liver slices. Mutation Research/Genetic Toxicology. Volume 371. Issues 3-4. Pages 273-282. **Acceptable/Non-Guideline** TXR 0058676	No evidence of induction of unscheduled DNA synthesis in human liver slices Dose Levels tested: and 0.05–2.5 mM PBO

Table 13. Mutation/Genotoxicity of PBO		
Guideline No. Study Type	**MRID No. (year) Classification**	**Results/Dose Levels**
870.5900 *In vitro* Sister Chromatid Exchange Assay NTP	00143499 (1983) **Acceptable/Guideline** TXR 0007674	The study does not indicate sister chromatid exchanges in CHO cells with and without metabolic activation.

C. Structure-Activity Relationship

According to the Compendium of Pesticide Common Names[4], PBO (Figure 5) is classified as a synergist. Along with PBO, other synergists (safrole (Figure 3), MGK-264 (Figure 4), and piperonyl sulfoxide (Figure 2)) were identified and screened for carcinogenicity. No relevant compounds were identified via the CompTox Dashboard[5] at ≥ 80% structural similarity, and most compounds above 60% similarity did not have identifiable and relevant data.

MGK-264 is classified as *Group C - Possibly Carcinogenic to Humans* based on statistically significant increases in hepatocellular adenomas in male and female mice and statistically significant increases in thyroid follicular cell adenomas in male rats (J. Doherty and E. Rinde, TXR 0011577, 06/07/1995). MGK-264, like PBO, inhibits mixed function oxidases (MFOs). Piperonyl sulfoxide was carcinogenic to male B6C3F1 mice, as determined by the NCI (DHEW Publication No.: NIH 79-1379), with liver carcinomas seen in both males and females. Safrole is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals (Report on Carcinogens, 15th edition, http://nt.nies.nih.gov/go/roc).

A Derek Nexus (v6.2.1) analysis was conducted for PBO and the other listed synergists. The relevant results include safrole producing three alerts of probable confidence (carcinogenicity, chromosomal damage, and non-specific genotoxicity). Studies conducted with safrole were the basis for the rules corresponding to the structural component and category of toxicity flagged above.

Overall, there is limited SAR support which identified other synergists, including MGK-264, piperonyl sulfoxide, and safrole, with structural similarities of <80%, which were also potentially carcinogenic.

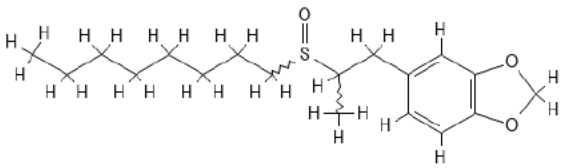

Figure 2: Structure of Piperonyl sulfoxide

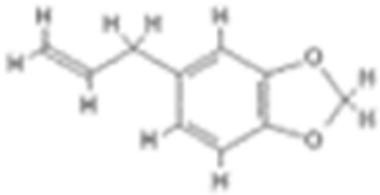

Figure 3: Structure of Safrole

[4] http://www.bcpcpesticidecompendium.org/
[5] https://comptox.epa.gov/dashboard/

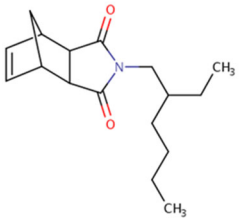

Figure 4 : Structure of MGK-264

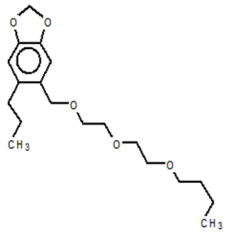

Figure 5: Structure of PBO

D. Other Subchronic and Chronic Toxicity Studies

1. Subchronic Toxicity

90-Day Oral Toxicity - Rat

In a 13-week oral toxicity non-guideline published study (MRID 43376307), F344 rats (10/sex/dose) were administered PBO technical grade in the diet at 0, 6000, 12000, or 24000 ppm (estimated dose 0, 600, 1200, 2400 mg/kg/day). At the end of experimental period, rats were necropsied. Selected organs were weighed, and serum was analyzed by clinical chemistry. In male and female rats in the 2400 mg/kg/day group, body weight gains were depressed, hepatomegaly was marked macroscopically, and liver weights were significantly higher than those of the control group. In male and female rats of all treated groups, relative kidney weights were significantly increased in a dose-dependent manner. Rats in the 2400 mg/kg/day dose group had increased levels of albumin, cholesterol, urea nitrogen, and gamma-glutamyl transpeptidase. Examination of livers of the males in the 2400 mg/kg/day dose group by light microscopy showed enlarged hepatocytes with glassy cytoplasm and fatty deposition. On occasion, there was coagulative necrosis of a few hepatocytes in the periportal area and oval cell proliferation. The kidney of treated rats showed atrophy of epithelium in the proximal convoluted tubules. These results indicated that toxicity of PBO in rats was directed primarily to the liver and kidney.

2. Chronic Toxicity

Rat

In a combined chronic toxicity/carcinogenicity study (MRID 40323701), groups of 60 Sprague-Dawley Crl:CDR (SD)BR rats/sex were administered PBO (87.67-89.71% a.i.; Reference # FEG32) in the diet at target dose levels of 0 (Group 1 control), 0 (Group 2 control), 30, 100, or 500 mg/kg/day for up to 104/105 (males/females) weeks. Additional groups of ten rats/sex/dose were included in the 0 (Group 2 control) and 30 mg/kg/day groups at the start of treatment, and 70 rats/sex were administered 15 mg/kg/day at the start of treatment. Ten animals/sex from the 0 (Group 2), 15, and 30 mg/kg/day groups were euthanized after treatment for four weeks and macroscopic/microscopic liver evaluations were conducted; the 15 mg/kg/day animals were discontinued on study by Week 8 due to lack of liver findings. Further evaluations (*i.e.*, body weight/body weight gain, food consumption, ophthalmology, hematology, clinical chemistry, urinalysis, organ weights, and macroscopic/microscopic pathology) were conducted on all surviving animals during the study and/or at scheduled termination.

There were no treatment-related effects on clinical signs, ophthalmoscopic findings, or hematology and urinalysis parameters.

No effects on body weights were seen at 30 or 100 mg/kg/day. At 500 mg/kg/day, body weights were decreased by 3-21% in both sexes from Weeks 1 through 104, with concomitant decreases in body weight gain observed during Weeks 0-24 and 24-52 in males (with body weight loss during Weeks 52-78) and during Weeks 0-24, 24-52, and 52-78 in females. Terminal body weights were significantly decreased (relative to both controls) by 21-23% and 20-31% in male and female rats at scheduled termination, respectively. The effects on body weight in both sexes were considered to be adverse.

Occasionally significant decreases in food consumption were observed in males from Week 72-73 and females from Week 57-58. Total food consumption in males was decreased by 10% during Weeks 79-104 and decreased by 7% each in females during Weeks 53-78 and 79-105 at 500 mg/kg/day. There was a treatment-related decrease of 5% in overall food consumption for females. The effects on food consumption were considered sporadic and not adverse.

Statistically significant treatment-related increases (relative to both controls) in total cholesterol were seen in females at ≥100 mg/kg/day at week 97/98 (93-111% at 100 mg/kg/day and 128-150% at 500 mg/kg/day) and at 500 mg/kg/day at week 51 (70-89%) and week 79 (80-90%). Treatment-related increases (relative to control Groups 1 and 2) were seen in total protein in females at 500 mg/kg/day at week 97/98 (13%) and in blood urea nitrogen (BUN) at Week 98 (93-121%).

Increased liver weights (absolute, relative to body weight and/or relative to brain weight) were seen in both sexes at weeks 97-105 relative to one or both controls at ≥30 mg/kg/day. For females surviving to weeks 104/105, liver weight changes included dose-related increases of 15-30% in absolute liver weights and 19-26% in relative (to brain) liver weight at ≥30 mg/kg/day, and increases of 23-76% in relative (to body weight) liver weights at ≥100 mg/kg/day. At 500 mg/kg/day, in surviving males at weeks 104/105, statistically significant liver weight (absolute/relative to body weight/relative to brain weight) increases (males: 19-24%/50-57%/22-28%) were noted relative to both controls. Hepatocyte hypertrophy was significantly increased in both sexes at 500 mg/kg/day. Observed treatment-related effects on the liver (*i.e.*, increased liver weight; increased incidences of enlarged and pale liver; and hepatocellular hypertrophy, with non-dose-related increases in focal hepatocellular hyperplasia and focal cell populations) along with increased clinical chemistry changes in cholesterol, total protein and/or BUN were considered to be adverse in females and not adaptive at this high dose. The increased liver weights seen at 30 and 100 mg/kg/day in females were not considered to be adverse since they were not accompanied by two or more enzymes or liver hypertrophy. The liver effects (increased liver weights and hypertrophy) in males were considered to be adaptive at ≥30 mg/kg/day since there were no sustained increased clinical chemistry parameters seen throughout the study.

In the thyroid, increased ($p<0.05$) incidences of pigment accumulation in the follicles of the 500 mg/kg/day males (48/60 treated vs. 24/60 and 27/60 controls) and females (44/60 treated vs. 8/60 and 10/60 controls) were observed, accompanied by increases ($p<0.05$) in thyroid follicular cell hyperplasia in the 30 and 500 mg/kg/day males (13/60 and 21/60 treated vs. 4/60 and 11/60 controls) and the ≥100 mg/kg/day females (9-11/60 treated vs. 0/60 and 4/60 controls). The dose related

increase in thyroid follicular cell hyperplasia observed in females at ≥100 mg/kg/day was considered to be adverse. Thyroid weights were not measured.

Mouse

In a carcinogenicity study (MRID 42903701*)*, groups of 60 CD-1® mice/sex were administered PBO (90.78% a.i.; Lot # FEP-100 12/12/89) via the diet at dose levels of 0 (group 1), 0 (Group 2), 30, 100, or 300 mg/kg/day for up to 78 weeks. Evaluations (*i.e.*, body weight/body weight gain, food consumption, hematology, organ weight, and macroscopic/microscopic pathology) were conducted on all surviving animals during the study and/or at scheduled termination.

There were no adverse, treatment-related effects on body weight/body weight gain, clinical signs or clinically observed palpable masses, food consumption, or hematology at any dose level in either sex.

In the liver, dose-dependent treatment-related increases (at $p<0.01$ or $p<0.05$) were observed in absolute liver weight/relative (to body) liver weight /relative (to brain) liver weight, respectively, at 100 mg/kg/day in males (↑19%/↑16%/↑20%, respectively) and in females (↑11%/↑8%/↑14%, respectively) and at 300 mg/kg/day in males (↑67%/↑67%/↑70, respectively) and in females(↑20%/↑19%/↑20%, respectively).

Treatment-related macroscopic pathology findings in the liver consisted of increased incidences of masses and nodules (combined) at ≥100 mg/kg/day in males (28-37/60 treated vs. 9-18/60 controls) and at 300 mg/kg/day in females (8/60 treated vs. 2/60 each control), increased size in the 300 mg/kg/day males (6/60 treated vs. 0-1 controls), and focal/multifocal color change in the 300 mg/kg/day males (13/60 treated vs. 4-8/60 controls).

Treatment-related non-neoplastic microscopic findings were limited to increased incidences of hepatocellular hypertrophy in males (43/60 treated vs. 6-11/60 controls) and females (9/60 treated vs. 0/60 Group 1 control only) and hemorrhage in males (13/60 treated vs. 1-2/60 controls) at 300 mg/kg/day and an increased incidence of hepatocellular hypertrophy in the 100 mg/kg/day males (16/60 treated vs. 6/60 Group 1 control only). Clinical chemistry was not measured in this study. Hepatocellular hyperplasia, considered to be adverse, was slightly increased at the high dose in both males (5/60 vs 0/120 controls) and females (4/60 vs 0/120 controls). In addition, the liver hypertrophy and hyperplasia was considered to be supportive of the liver tumors seen in males at ≥100 mg/kg/day and in females at 300 mg/kg/day.

IV. PROPOSED MODE OF ACTION FOR LIVER TUMORS IN THE MOUSE

A. Proposed Mode of Action for Liver Tumors in Male and Female Mice

PBTFII submitted a White Paper (MRID 51692501) to assess the PBO-induced hepatocarcinogenicity in the mouse and to propose a mitogenic MOA involving constitutive androstane receptor (CAR) activation for mouse liver tumor formation using the framework developed by the International Programme on Chemical Safety (IPCS)/World Health Organization (WHO)/International Life Sciences Institute (ILSI) working groups (Boobis *et al.* 2006; Cohen *et al.* 2003; Meek *et al.* 2003, 2014a,b). In

order to elucidate a MOA for PBO-induced mouse liver tumor formation, the PBTFII conducted a series of mechanistic studies in male mice. In the first series of 7- and 14-day studies, the effects of PBO on markers of liver hypertrophy, replicative DNA synthesis (RDS), apoptosis, and induction of cytochrome P450 enzymes (CYPs) and other enzymes were determined in male CD-1 mice given dose levels of 30, 100 or 300 mg/kg/day of PBO (MRID 51692502, MRID 51692503). As stated in the PBTFII white paper, male mice were selected for these studies as they are more susceptible to PBO-induced liver tumor formation than female mice. According to Peffer *et al*. 2018, "The most compelling MOA data will be those generated in the same species, strain, and sex of animal in which the tumors were observed following lifetime treatment with the test compound. If practical, the same test diet and age/source of animal should also be used in the mechanistic studies. In cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient." As a positive control, male mice were also given 500 ppm in the diet of sodium phenobarbital, this compound being a known CAR activator in mouse liver (Elcombe *et al*., 2014; Lake, 2018; Yamada *et al*., 2021). Recovery studies were also included in these investigations. In the second series of 7- and 14-day studies, the effects of PBO on markers of liver hypertrophy, RDS, and induction of CYPs and other enzymes was investigated in male C57BL/6J (wild type) and in hepatic CAR and pregnane X receptor (PXR) knockout (CAR KO/PXR KO) mice (MRID 51692504, MRID 51692505). The commercially available CAR KO/PXR KO mice were bred on a C57BL/6J background (Scheer *et al*., 2008), hence the use of C57BL/6J mice as the control (wild type) mice for these investigations. In a final series of studies to assess the applicability of the mouse liver tumor MOA for humans, the effect of PBO and sodium phenobarbital on RDS in cultured male CD-1 mouse and male and female human hepatocytes was investigated (MRID 51692506 and 51692507).

List of Submitted Information:

1. (MRID 51692501): Lake, B. and Osmitz, T. 2021. Prepared for the Piperonyl Butoxide Task Force II (PBTFII). Mode of Action (MOA) Analysis for Mouse Liver Tumor Formation by Piperonyl Butoxide (PBO) and Human Relevance

2. (MRID 51692502): Hosako, H. 2015. Piperonyl Butoxide – Mode of Action (MOA) Phase 1: A 14-Day Dietary Study Comparing the Hepatic Effects of Piperonyl Butoxide and Sodium Phenobarbital in Male CD-1 Mice (Volume 1 of 3)

3. (MRID 51692503): Lake, B.G. 2012. A 14-Day dietary study comparing the hepatic effects of piperonyl butoxide and sodium phenobarbital in male CD-1 mice. Analytical phase report for liver biochemistry analysis

4. (MRID 51692504): Bowers, M. 2014. Piperonyl Butoxide (PBO) – Dietary 7/14-Day Study to Investigate the Hepatic Effects of PBO in Male Constitute Androstane Receptor (CAR)/Pregnane X Receptor (PXR) Double Knockout and Wild Type Mice

5. (MRID 51692505): Lake, B.G. 2013. Comparison of the hepatic effects of piperonyl butoxide in male CAR)/PXR double knockout and wild type C57BL/6J mice

6. (MRID 51692506): Elcombe, B. and Vardy, A. 2017. Piperonyl Butoxide: MOA Phase III – Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male CD-1 Mouse Hepatocytes

7. (MRID 51692507): Elcombe, B. and Vardy, A. 2017. Piperonyl Butoxide: MOA Phase III – Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male and Female Human Hepatocytes

As part of the current review, a survey of the literature was conducted by EPA to identify any additional toxicological or mechanistic studies relevant to the proposed MOA for liver adenomas and carcinomas in mice exposed to PBO. Two mechanistic studies that provided additional support for several key and associative events were identified. The Philips *et al* 1997 study, identified in the literature search, was also submitted by the registrant. (See Appendix A for details on the literature search).

Sakamoto, Y., Inoue, K., Takahashi, M., *et al.* (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. *Toxicol. Pathol.* 41, pp. 1078-1092. MRID 52376003.

Phillips, J.C., Price, R.J., Cunninghame, M.E., *et al.* (1997) Effect of piperonyl butoxide on cell replication and xenobiotic metabolism in the livers of CD-1 mice and F344 rats. *Fund. Appl. Toxicol.* 38(1), 64-74. MRID 52376008.

The following postulated MOA for the PBO induced liver tumors involves the following Key and Associative Events (**Figure 6**):

Key Event #1: Activation of hepatic constitutive androstane receptor (CAR)
Key Event #2: Increased cell proliferation
Associative Event #1: Increased liver weight[6]
Associative Event #2: Liver hypertrophy
Key Event #3: Clonal expansion leading to altered liver foci
Key Event #4: Liver tumor formation

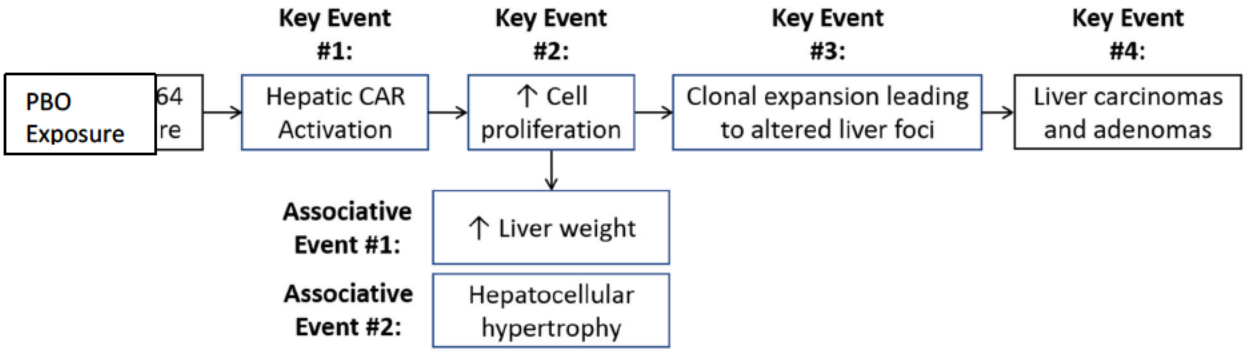

Figure 6. Proposed MOA for Liver Tumor Formation in Mice Resulting from Exposure to PBO

The key and associative events for the proposed MOA for PBO-induced mouse liver tumor formation are detailed below and have been evaluated using the modified Bradford Hill considerations (Boobis *et al*., 2006; Meek *et al*., 2003) and in accordance with the EPA's *Guidelines for Carcinogen Risk Assessment* (March 2005). The CARC considered the hepatocarcinogenic treatment related dose levels to be ≥100 mg/kg/day in male CD-1 mice and 300 mg/kg/day in female CD-1 mice.

The data supporting each key event are described in independent sub-sections, each concluding with a summary specific for that key event.

<u>Key event #1: Activation of hepatic CAR</u>

Overview: The ability of PBO to activate CAR was examined in two mechanistic studies (MRIDs 51692503 & 51692505). In these studies, activation of CAR was evaluated indirectly through the induction of Cyp2b expression and Cyp2b enzyme activity, hepatic Cyp2b10 mRNA levels, microsomal 7-pentoxyresorufin *O*-depentylase (PROD) activity and Cyp2b protein content. The role of hepatic CAR was also demonstrated in the study with C57BL/6J (wild type) and CAR KO/PXR KO mice (i.e. in mice lacking hepatic CAR and PXR), where PBO markedly induced Cyp2b enzyme activity and Cyp2b10 mRNA levels in C57BL/6J mice, but only produced a small increase in microsomal PROD activity and a significant decrease in hepatic Cyp2b10 mRNA levels in CAR KO/PXR KO mice. In another mechanistic study (MRID 52376003), the induction of male mouse hepatic Cyp2b10 mRNA levels was demonstrated

[6] The Registrant's white paper notes Associative Events #1 and #2 as liver hypertrophy and induction of Cyp2b enzymes, respectively. However, the Agency has slightly modified the Associative Events to be consistent with other chemicals the Agency has evaluated for this MOA.

with C3H/HeNCrlCrlj (wildtype) and CAR KO mice.

1. Lake, B.G. (2012) A 14-day dietary study comparing the hepatic effects of piperonyl butoxide and sodium phenobarbital in male CD-1 mice. Analytical phase report for liver biochemistry analysis. WIL Research Laboratories, LLC, Ashland, OH. WIL Study No.: WIL-782006, September 12, 2012. Unpublished. MRID 51692503.

In a non-guideline, *in vitro* liver tumor MOA study (MRID 51692503), frozen liver samples were prepared in a previous *in vivo* study (MRID 51692502) from groups of 8 CD-1 [Crl:CD1(ICR)] male mice that were administered PBO (93.8% a.i.; Lot # E104/10) in the diet at concentrations of 0, 30, 100, and 300 mg/kg/day for 14 days with or without a 28-day recovery period. A positive control, 0.05% sodium phenobarbital (NaPB), was run concurrently. A recovery phase included control, high-dose, and positive control groups only. In this *in vitro* study, liver whole homogenates were assayed for protein content and for cyanide-insensitive palmitoyl-CoA oxidation (PCoA) activity as a marker of the peroxisomal fatty acid β-oxidation cycle (Lake, 2009). Liver microsomes were assayed for protein and total cytochrome CYP content and for 7-ethoxyresorufin O-deethylase (EROD), 7-pentoxyresorufin O-dependentylase (PROD), testosterone 6β-hydroxylase (T6βOH) and lauric acid 12-hydroxylase (LA12OH) activities as markers for induction of Cyp1a, Cyp2b, Cyp3a, and Cyp4a subfamily enzymes, respectively. Liver samples were also assayed for Cyp1a2, Cyp2b10, Cyp3a11, and Cyp4a10 mRNA levels and liver microsomes were assayed for Cyp1a, Cyp2b, Cyp3a, and Cyp4a protein levels.

Summary: The results indicated that the hepatic effects of PBO were similar to NaPB (a known CAR activator/positive control), which suggests that PBO is a CAR activator in mice. Microsomal protein content was significantly increased by treatment with 100 and 300 mg/kg/day PBO, with 30-300 mg/kg/day PBO producing statistically significant dose-dependent increases in microsomal total CYP content and in PROD enzyme activity. Treatment with PBO also produced dose-dependent increases in microsomal EROD and T6βOH activities, although the greatest effect was on PROD activity. The treatment of male CD-1 mice with PBO had no significant effect on whole homogenate protein content, whereas whole homogenate PCoA activity was significantly decreased by treatment with 30 and 300 mg/kg/day PBO, indicating PBO did not produce any obvious effects on the markers of peroxisome proliferation.

Table 14 presents an overall summary of results from **Tables 15-16**. In the study (without recovery), microsomal protein content was increased at ≥100 mg/kg/day (↑11-23%) and in the positive control group (↑27%), and total CYP content was increased at ≥30 mg/kg/day (↑21-72%) and in the positive control group (↑132%) (See **Table 15** below for detailed information). Microsomal EROD and PROD activities were increased at ≥30 mg/kg/day (↑40-145% and ↑65-329%, respectively) and in the positive control group (↑217% and ↑1132%, respectively), and testosterone 6β-hydroxylase activity was increased at ≥100 mg/kg/day (↑60-175%) and in the positive control group (↑84%) (See **Table 16** below for detailed information).

Table 14. Effect of dietary administration of 30, 100, and 300 mg/kg/day PBO and 500 ppm NaPB to male CD-1 mice for 14 days and treatment of male CD-1 mice with 300 mg/kg/day PBO and 500 ppm NaPB for 14 days followed by 28 days on recovery on some hepatic parameters (Results are presented as % of control levels).

Parameter[a]	14 Day study[b,c]				Recovery study[b,c]	
	PBO 30 mg/kg/day	PBO 100 mg/kg/day	PBO 300 mg/kg/day	NaPB 500 ppm	PBO 300 mg/kg/day	NaPB 500 ppm
Relative liver weight	91*	108*	129**	121**	100	105
Whole homogenate protein	103	102	100	97	97	99
Whole homogenate PCoA activity	73*	93	69*	50***	107	121
Microsomal protein content	97	111*	123***	127***	99	107
Microsomal total CYP content	121*	145***	172***	232***	110	113
Microsomal EROD activity	140*	168***	245***	317***	102	98
Microsomal PROD activity	165*	335***	429***	1232***	163**	153
Microsomal T6βOH activity	115	160***	275***	184***	95	94
Microsomal LA12OH activity	88	152**	84	61*	96	157

[a] Relative liver weight expressed as g liver/100 g body, microsomal protein content as mg/g liver, CYP content as nmol/mg protein, and enzyme activity as nmol or pmol/min/protein.

[b] n= 8 mice.

[b] Table from MRID 51692501 extracted from Lake (2012) [MRID 51692503].

* Significantly different from control; $p<0.5$;

** Significantly different from control; $p<0.01$;

*** Significantly different from control; $p<0.001$.

Table 15. Mean (± SD) microsomal protein (mg/g liver) and CYP (nmol/mg protein) content in male mice administered PBO in the diet for 14 days with or without a 28-day recovery period.[a]

Parameter	Dose (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
Without recovery period					
Microsomal protein content	36.6 ± 5.01	35.4 ± 3.93	40.6 ± 1.91* (↑11%)	44.9 ± 4.04*** (↑23%)	46.6 ± 4.50*** (↑27%)
CYP content	0.47 ± 0.079	0.57 ± 0.084* (↑21%)	0.68 ± 0.069*** (↑45%)	0.81 ± 0.113*** (↑72%)	1.09 ± 0.138*** (↑132%)
With recovery					
Microsomal protein content	31.4 ± 2.58	-	-	31.0 ± 2.08	33.7 ± 1.96
CYP content	0.48 ± 0.116	-	-	0.53 ± 0.106	0.54 ± 0.095

a Data were obtained from Table 2 on page 32 and Table 8 on page 38 of MRID 51692503; n = 8.

- No data.

* Significantly different from control; p<0.05;

*** Significantly different from control; p<0.001.

Table 16. Mean (± SD) microsomal CYP-dependent enzyme activity in male mice administered PBO in the diet for 14 days with or without a 28-day recovery period. [a]

Parameter	Dose (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
Without recovery period					
EROD (pmol/min/mg protein)	47 ± 14.2	66 ± 8.8* (↑40%)	79 ± 16.8*** (↑68%)	115 ± 17.7*** (↑145%)	149 ± 14.1*** (↑217%)
PROD (pmol/min/mg protein)	31 ± 9.1	51 ± 10.9* (↑65%)	104 ± 20.3*** (↑235%)	133 ± 25.1*** (↑329%)	382 ± 68.2*** (↑1132%)
Testosterone 6β-hydroxylase (nmol/min/mg protein)	1.02 ± 0.214	1.17 ± 0.190	1.63 ± 0.286*** (↑60%)	2.80 ± 0.260*** (↑175%)	1.88 ± 0.199*** (↑84%)
Lauric acid 12-hydroxylase (nmol/min/mg protein)	2.29 ± 0.752	2.01 ± 0.805	3.48 ± 0.580** (↑52%)	1.92 ± 0.318	1.40 ± 0.407* (↓39%)
With recovery					
EROD (pmol/min/mg protein)	59 ± 11.8	-	-	60 ± 8.5	58 ± 11.0
PROD (pmol/min/mg protein)	19 ± 7.5	-	-	31 ± 7.5** (↑63%)	29 ± 14.1 (↑53%)
Testosterone 6β-hydroxylase (nmol/min/mg protein)	1.11 ± 0.177	-	-	1.06 ± 0.090	1.04 ± 0.141
Lauric acid 12-hydroxylase (nmol/min/mg protein)	1.15 ± 0.686	-	-	1.10 ± 0.307	1.80 ± 0.882

a Data were obtained from Tables 3-4 on page 33-34 and Tables 9-10 on pages 39-40 of MRID 51692503; n = 8.
\- No data.
* Significantly different from control; p<0.05.
** Significantly different from control; p<0.01.
*** Significantly different from control; p<0.001.

As shown in **Tables 17 and 18**, there were dose-related increases in mRNA expression levels of CYP1A2 and CYP2B10 at ≥30 mg/kg/day (1.4- to 3.5-fold and 1.6- to 17-fold, respectively) and in the positive control (3.8-fold and 30-fold, respectively), and CYP3A11 at ≥100 mg/kg/day (1.7- to 5.1-fold) and in the positive control (2.8-fold) (**Table 17**). There were associated increases in protein levels of CYP1A at ≥30 mg/kg/day (1.3- to 3.1-fold) and in the positive control (3.3-fold), CYP2B at ≥100 mg/kg/day (2.5- to 5.4-fold) and in the positive control (4.8-fold), and CYP3A at 300 mg/kg/day (1.8-fold) and in the positive control (1.5-fold) (**Table 18**). In the recovery study, there was generally a complete recovery of protein content and CYP induction markers observed during the main study. The only remaining effects included increases in microsomal PROD activity at 300 mg/kg/day (↑63%) and in the positive control group (↑53%) and an increase in the CYP3A protein level at 300 mg/kg/day, although to a lesser degree than the main study (1.3-fold).

Overall, the hepatic effects of PBO were qualitatively similar to those of NaPB in that both compounds produced a marked induction of Cyp2B forms and to a lesser extent of markers of Cyp1A and Cyp3A forms.

There were no dose-related changes in markers of peroxisome proliferation (Cyp4A10) in any PBO treatment. Treatment of male CD-1 mice with 30-300 mg/kg/day for 14 days did not have any obvious

effect on the markers of hepatic peroxisome proliferation, including whole homogenate palmitoyl-CoA oxidation activity (**Table 19**), microsomal lauric acid 12-hydroxylase activity (**Table 16**), microsomal Cyp4A protein levels (**Table 18**), and hepatic Cyp4A10 mRNA levels (**Table 17**). While small statistically significant increases in microsomal lauric acid 12-hydroxylase activity, microsomal Cyp4A protein levels and hepatic Cyp4A10 mRNA levels were observed at 100 mg/kg/day PBO, treatment with 300 mg/kg/day PBO led to a statistically significant reduction in whole homogenate palmitoyl-CoA oxidation activity, microsomal Cyp4A protein levels and hepatic Cyp4A10 mRNA levels. This demonstrates that overall, the MOA for PBO induced mouse liver tumors is unlikely to be associated in PPARα activation.

Table 17. Mean (± SD) hepatic CYP mRNA levels (fold induction) in male mice administered PBO in the diet for 14 days with or without a 28-day recovery period. [a]

Parameter	Dose (mg/kg/day)				
	0	30	100	300 [b]	NaPB (0.05%)
Without recovery period					
CYP1A2	1.00 ± 0.192	1.40 ± 0.269**	2.29 ± 0.449*** [b]	3.50 ± 1.242**	3.82 ± 0.828***
CYP2B10	1.00 ± 0.120	1.63 ± 0.714*	4.27 ± 1.041***	16.97 ± 8.358***	30.25 ± 11.090***
CYP3A11	1.00 ± 0.118	1.39 ± 1.032	1.67 ± 0.398**	5.07 ± 3.237*	2.83 ± 1.367**
CYP4A10	1.00 ± 0.270	1.12 ± 0.966	1.80 ± 0.709*	0.37 ± 0.318**	0.57 ± 0.248**
With recovery					
CYP1A2	1.00 ± 0.116	-	-	0.84 ± 0.298	0.84 ± 0.174*
CYP2B10	1.00 ± 0.056	-	-	0.52 ± 0.229**	0.62 ± 0.421*
CYP3A11	1.00 ± 0.437	-	-	0.69 ± 0.285	0.88 ± 0.190
CYP4A10	1.00 ± 0.246	-	-	0.88 ± 0.329	3.09 ± 3.090

a Data were obtained from Table 5 on page 35 and Table 11 on page 41 of MRID 51692503; n = 8, unless otherwise noted.

b n = 7.

- No data.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

Table 18. Mean (± SD) microsomal CYP protein levels (fold induction) in male mice administered PBO in the diet for 14 days with or without a 28-day recovery period. [a]

Parameter	Dose (mg/kg/day)					
	0	30	100	300	0	NaPB (0.05%)
Without recovery period						
CYP1A	1.00 ± 0.240	1.32 ± 0.207**	1.84 ± 0.109***	3.11 ± 0.584***	1.00 ± 0.233	3.32 ± 0.423***
CYP2B	1.00 ± 0.434[b]	1.33 ± 0.291	2.51 ± 1.307***	5.39 ± 2.649***	1.00 ± 0.447	4.77 ± 1.221***
CYP3A	1.00 ± 0.420	0.95 ± 0.165	1.40 ± 0.401	1.75 ± 0.665**	1.00 ± 0.455	1.52 ± 0.463*
CYP4A	1.00 ± 0.437	0.84 ± 0.460	1.39 ± 0.315*	0.41 ± 0.117**	1.00 ± 0.442	0.44 ± 0.156**
With recovery						
CYP1A	1.00 ± 0.205	-	-	0.98 ± 0.260	-	1.20 ± 0.606
CYP2B	1.00 ± 0.347	-	-	1.21 ± 0.366	-	1.44 ± 0.697
CYP3A	1.00 ± 0.189	-	-	1.25 ± 0.211*	-	1.51 ± 0.697
CYP4A	1.00 ± 0.565	-	-	0.96 ± 0.222	-	1.88 ± 1.438

a Data were obtained from Table 6 on page 36 and Table 12 on page 42 of MRID 51692503; n = 8, unless otherwise noted.
b n = 7.
- No data.
* Significantly different from control; p<0.05.
** Significantly different from control; p<0.01.
*** Significantly different from control; p<0.001.

Table 19. Mean (± SD) whole homogenate protein content (mg/g liver) and palmitoyl Co-A oxidation activity (nmol/min/mg protein) in male mice administered PBO in the diet for 14 days with or without a 28-day recovery period. [a]

Parameter	Dose (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
Without recovery period					
Whole homogenate protein	223 ± 14.3	230 ± 6.3	228 ± 10.7	223 ± 5.1	216 ± 8.2
Palmitoyl-CoA oxidation	10.5 ± 1.46	7.7 ± 1.79* (↓27%)	9.8 ± 2.52	7.2 ± 3.83* (↓31%)	5.3 ± 0.83*** (↓50%)
With recovery					
Whole homogenate protein	220 ± 7.9	-	-	214 ± 5.5	217 ± 7.7
Palmitoyl-CoA oxidation	9.2 ± 3.12	-	-	9.8 ± 2.50	11.1 ± 2.98

a Data were obtained from Table 1 on page 31 and Table 7 on page 37 of MRID 51692503; n = 8.
- No data.
* Significantly different from control; p<0.05.
*** Significantly different from control; p<0.001.

2. Constitutive androstane receptor (CAR)/pregnane X receptor (PXR) double knockout and wild type mice

Lake, B.G. (2013) Comparison of the hepatic effects of piperonyl butoxide in male constitutive androstane receptor (CAR)/pregnane X receptor (PXR) double knockout and wild type C57BL/6J mice.

Leatherhead Food Research, Molecular Sciences Department, Surrey, UK. Laboratory Study No.: 5507/1, October 24, 2013. Unpublished. MRID 51692505.

The aim of this series of studies was to evaluate the hepatic effects of PBO in normal (i.e. wild type) mice and in mice lacking hepatic CAR. Because there can be considerable crosstalk between the hepatic CAR and PXR receptors, with some compounds being activators of both these nuclear receptors (Omiecinski *et al*., 2011; Maglich *et al*., 2002; Yoshinari *et al*., 2008), mice lacking both hepatic CAR and PXR receptors (i.e. CAR and PXR knockout (KO) mice) were selected for these studies. As the commercially available CAR KO/PXR KO mice used in these studies were bred on a C57BL/6J background (Scheer et al., 2008), this mouse strain was employed as the control (i.e. wild type) mice for these investigations. This strain was a different background strain than was used in the guideline study (MRID 42903701).

In a non-guideline, *in vitro* liver tumor MOA study (MRID 51692505), liver tissue samples from groups of 8 male C57BL/6J (wild type) and 8 male PXR KO/CAR KO mice administered PBO in the diet for 14 days at target dose levels of 0 and 300 mg/kg/day (mean achieved intakes of 0 and 291 mg/kg/day for C57BL/6J mice and 0 and 236 mg/kg/day for PXR KO/CAR KO mice) were evaluated. Whole liver homogenates and microsomal fractions were evaluated for protein content; liver homogenates were evaluated for palmitoyl-CoA oxidation activity; and microsomal fractions were evaluated for select cytochrome P450 (CYP)-dependent enzyme activities, CYP mRNA levels, and CYP protein levels.

Wild type mice. Microsomal EROD and PROD activities were increased by 6-fold and 13.7-fold, respectively, and testosterone 6β-hydroxylase and lauric acid 12-hydroxylase activities were increased by 140% and 106%, respectively (**Table 20**). The mRNA levels of CYP1A2, CYP2B10, and CYP3A11 were increased by 3.95-fold, 1297-fold, and 8.90-fold, respectively (**Table 21**). Protein levels for CYP1A, CYP2B, CYP3A, and CYP4A were increased by 4-fold, 5.76-fold, 3.05-fold, and 1.37-fold, respectively (**Table 22**). In hepatic microsomal fractions from wild type mice administered PBO for 14 days, the microsomal protein level was increased by 27%, and total CYP content was increased by 135% (**Table 23**).

KO Mice. Microsomal EROD and PROD activities were increased by 41% and 50%, respectively, testosterone 6β-hydroxylase activity was decreased ($p<0.001$) by 72%, and lauric acid 12-hydroxylase activity was increased by 120% (**Table 20**). The mRNA levels of acyl-CoA oxidase, CYP1A2, and CYP4A10 were increased by 73%, 416%, and 350%, respectively, and the mRNA level of CYP2B10 was decreased by 61% (**Table 21**). Protein levels for CYP1A, CYP2B, and CYP4A were increased by 363%, 73%, and 112%, respectively (**Table 22**). In whole liver homogenates from CAR KO/PXR KO mice administered PBO for 14 days, palmitoyl-CoA oxidation activity was increased by 78% (**Table 23**). In hepatic microsomal fractions from the CAR KO/PXR KO mice, total CYP content was decreased by 23% (**Table 23**).

Table 20. Mean (± SD) CYP-dependent enzyme activity in microsomal fractions derived from livers of male mice administered PBO in the diet for 14 days. [a]

Parameter	Dose (mg/kg/day)			
	C57BL/6J (wild type)		PXR KO/CAR KO	
	0	300	0	300
7-ethoxyresorufin O-deethylase (EROD) (pmol/minute/mg protein)	41 ± 7.6	265 ± 45.1*** (↑546%)	71 ± 14.3	100 ± 7.4*** (↑41%)
7-pentoxyresorufin O-dependentylase (PROD)(pmol/minute/mg protein)	10 ± 2.4	137 ± 20.9*** (↑1270%)	14 ± 1.7	21 ± 4.8** (↑50%)
Testosterone 6β-hydroxylase (nmol/minute/mg protein)	0.25 ± 0.043	0.60 ± 0.066*** (↑140%)	0.46 ± 0.089	0.13 ± 0.030*** (↓72%)
Lauric acid 12-hydroxylase (nmol/minute/mg protein)	0.85 ± 0.141	1.75 ± 0.320[b]*** (↑106%)	0.75 ± 0.189	1.65 ± 0.185*** (↑120%)

[a] Data were obtained from Tables 3 and 4 on pages 28-29 of MRID 51692505; n = 8.

[b] n=7.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

Table 21. Mean (± SD) mRNA levels (fold change) in microsomal fractions derived from livers of male mice administered PBO in the diet for 14 days. [a]

mRNA	Dose (mg/kg/day)			
	C57BL/6J		PXR KO/CAR KO	
	0	300	0	300
Acyl-CoA oxidase	1.00 ± 0.167	1.07 ± 0.323	1.00 ± 0.150	1.73 ± 0.370***
Cyp1a2	1.00 ± 0.133	3.95 ± 0.878***	1.00 ± 0.148	5.16 ± 1.258***
Cyp2b10	1.00 ± 0.088	1297 ± 444.0***	1.00 ± 0.091	0.39 ± 0.420**
Cyp3a11	1.00 ± 0.292	8.90 ± 5.465**	1.00 ± 0.374	1.56 ± 0.664
Cyp4a10	1.00 ± 0.164	1.32 ± 0.823	1.00 ± 0.243	4.50 ± 1.552***

a Data were obtained from Table 5 on page 30 of MRID 51692505; n = 8.

** Significantly different from control; p<0.01.

*** Significantly different from control; p<0.001.

Table 22. Mean (± SD) CYP protein levels (fold change) in microsomal fractions derived from livers of male mice administered PBO in the diet for 14 days. [a]				
CYP protein	Dose (mg/kg/day)			
	C57BL/6J		PXR KO/CAR KO	
	0	300	0	300
Cyp1a	1.00 ± 0.456	4.03 ± 0.692***	1.00 ± 0.281	4.63 ± 1.183***
Cyp2b	1.00 ± 0.333	5.76 ± 1.115***	1.00 ± 0.353	1.73 ± 0.325***
Cyp3a	1.00 ± 0.318	3.05 ± 0.731***	1.00 ± 0.367	0.97 ± 0.199
Cyp4a	1.00 ± 0.189	1.37 ± 0.203**	1.00 ± 0.295	2.12 ± 0.127***

a Data were obtained from Table 6 on page 31 of MRID 51692505; n = 8.
** Significantly different from control; p<0.01.
*** Significantly different from control; p<0.001.

Table 23. Mean (± SD) palmitoyl CoA oxidation activity and total CYP content in whole liver homogenates and microsomal fractions derived from livers of male mice administered PBO in the diet for 14 days.[a]				
Parameter	Dose (mg/kg/day)			
	C57BL/6J		PXR KO/CAR KO	
	0	300	0	300
Whole liver homogenate				
Palmitoyl-CoA oxidation (nmol/minute/mg protein)	8.2 ± 0.89	7.7 ± 1.31	9.3 ± 2.19	16.6 ± 2.60*** (↑78%)
Whole liver homogenate protein (mg/g liver)	198±8.4	201±6.9	203±7.4	204±5.2
Microsomal fraction				
Total cytochrome P450 (nmol/mg protein)	0.23 ± 0.033	0.54 ± 0.063*** (↑135%)	0.26 ± 0.036	0.20 ± 0.022*** (↓23%)
Microsomal protein (mg/g liver)	27.9±1.56	35.5±4.26** (↑27%)	30.5±2.18	32.4±2.61

a Data were obtained from Tables 1 and 2 on pages 26-27 of MRID 51692505; n = 8.
*** Significantly different from control; p<0.001.
** Significantly different from control; p<0.01.

3. Sakamoto, Y., Inoue, K., Takahashi, M., et al. (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. Toxicol. Pathol. 41, pp. 1078-1092. MRID 52376003.

The induction of mouse hepatic Cyp2b10 mRNA levels has also been demonstrated by Sakamoto *et al.*, 2013. In a carcinogenicity mechanism of action study, groups of male C3H/HeNCrlCrlj (wild-type) or C3H/HeNCrl background constitutive androgen receptor knockout (CARKO) mice were administered PBO (>90% a.i.; Lot # not provided), decabromodiphenyl ether (DBDE), or sodium phenobarbital (NaPB) in the diet at concentrations of 5000 ppm (~1000 mg/kg/day), 50,000, or 500 ppm, respectively, for 1, 4, or 27 weeks; groups of five animals/treatment/genotype (wild-type or CARKO) were used in the short-term experiments, and 20 wild-type or 21-24 CAR KO mice/treatment were used for the 27-week experiment. [It is noted that the KO mouse strain used in this study was different than the one used in the guideline study (MRID 42903701; CD-1 mouse) and in MRID 51652505

(C57BL/6J mouse background strain)]. PBO and DBDE were used in this experiment because they are both Cyp2b-inducing nongenotoxic hepatocarcinogens. DBDE, a brominated flame retardant, is a known PXR activator and induces Cy2b and Cyp3a but not Cyp1a1. NaPB is frequently used as a positive control for Cyp inducers, particularly Cyp2b. Gadd45 can be induced by a distinctive pathway that requires CAR. Increased levels of Gadd45 beta mRNA may imply the resistance of apoptosis.

In wild-type mice, treatment with PBO for 4 weeks increased hepatic mRNA expression levels of *Cyp2b10 > Gadd45beta > Cyp3a11 > CYP reductase > Cyp1a2*; treatment with DBDE increased hepatic mRNA expression levels of *Cyp1a1 > Cyp2b10 > Cyp1b1 > Cyp1a2 > Cyp3a11 > CYP reductase;* and treatment with NaPB increased hepatic mRNA expression levels of *Cyp2b10 > Gadd45beta > CYP reductase > Cyp1a2 ≈ Cyp3a11 > Cyp1a1.*

In CAR KO mice, treatment with PBO for 4 weeks increased hepatic mRNA expression levels of *Cyp4a10 > Cyp2b10 > Gadd45beta > Cyp1a1 > Cyp3a11 ≈ CYP reductase > Cyp1a2*; treatment with DBDE increased hepatic mRNA expression levels of *Cyp1a1 > Cyp1b1 > Cyp1a2 > CYP reductase;* and treatment with NaPB increased hepatic mRNA expression levels of *Cyp3a11.*

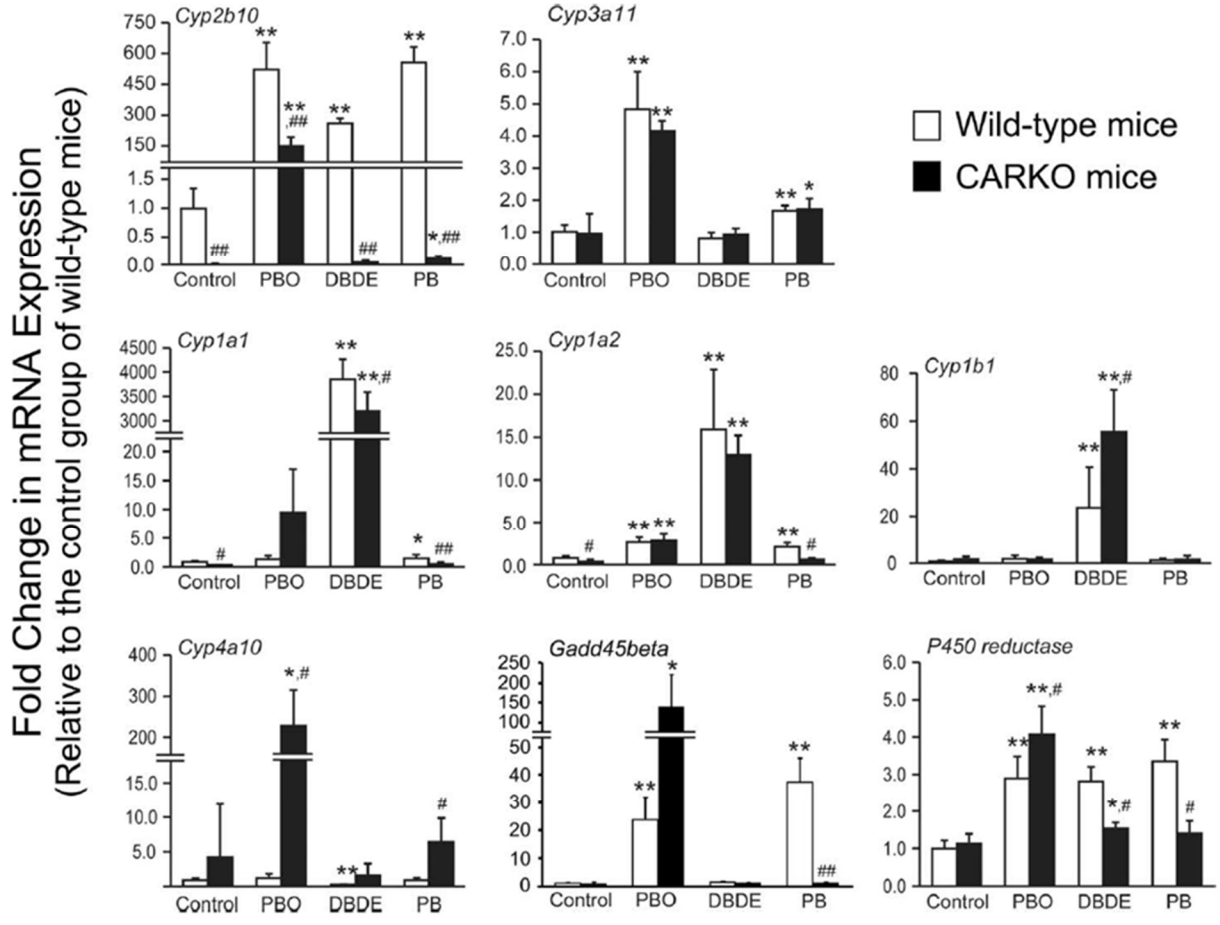

FIGURE 3.—Relative mRNA expression levels of *Cyp2b10, Cyp3a11, Cyp1a1, Cyp1a2, Cyp1b1, Cyp4a10, Gadd45beta,* and *P450 reductase* in the liver of wild-type and CARKO mice treated with test chemicals for 4 weeks. Values represent *Mean* ± *SD* of each group and genotype. The expression levels of each gene were divided by the expression levels of *GAPDH* mRNA of corresponding individuals. * and ** indicate statistically significant differences from the control group of each genotype (*$p < .05$, **$p < .01$: Student and Welch test). # and ## indicate statistically significant differences from the wild-type animals of each group ($^{\#}p < .05$, $^{\#\#}p < .01$: Student and Welch test).

Figure 7: Relative mRNA Expression Levels

CARC Conclusions on Key Event #1 *(CAR activation and enzyme induction in the liver)*: *The CARC determined that key event #1 (CAR activation and enzyme induction in the liver) is adequately supported. Statistically significant and dose-related increases in Cyp2b10 mRNA levels, PROD enzyme activity, and total P-450 protein levels were seen in male mice at 30-300 mg/kg/day PBO after 2 weeks of exposure, including robust increases (ranging from 2-17-fold induction) at the tumorigenic doses of 100 and 300 mg/kg/day. Male mice were selected for these studies as they are more susceptible to PBO-induced liver tumor formation than female mice and the MOA is considered to be the same in both sexes. While PBO also has some effects on mouse hepatic Cyp1a, Cyp3a and Cyp4a subfamily enzymes, the induction of the Cyp2b markers was greater than that of the Cyp1a, Cyp3a and Cyp4a markers. No dose-related changes were seen in Cyp4a10 induction, indicating suppression of PPAR-α-mediated signaling in the liver. While induction of Cyp2B is an indicator of CAR activation, PXR activation can also produce an induction of Cyp2B enzymes, along with a greater induction of CYP3A enzymes. Because there can be considerable crosstalk between the hepatic CAR and PXR receptors, with some compounds being activators of both of these nuclear receptors, male mice lacking both hepatic CAR and PXR receptors (i.e. CAR and PXR KO mice) were evaluated in mechanistic studies with 300 mg/kg/day PBO for 14 days. The CARKO/PXRKO data presented further supported specificity for activation of the CAR/PXR receptor. There was little to no increase in Cyp4a10 markers, indicative of PPAR-α activation, with wildtype mice, with a stronger increase in the Cy4a10 markers in CAR/PXR KO mice. The increased Cyp1a2 markers, indicative of aromatic hydrocarbon-responsive receptor (AhR) activation, seen in CD-1 mice as well as the wildtype and CAR/PXR KO mice is likely due to crosstalk. A study by Ryu et al. 1996 showed that AhR knockout mice treated with 200 mg/kg PBO demonstrated increased Cyp1a2 induction (increased Cyp1a2 mRNA levels in AhR KO mouse liver), without the induction of Cyp1a1, which suggests the possibility of an AhR-independent mechanism of Cyp1a2 induction, likely due to crosstalk. Additional evidence for crosstalk among receptors was seen in short-term (4-week) treatment using a very high dose (1000 mg/kg/day) of PBO in wildtype and CAR KO mice, with the most robust response for Cyp2b induction seen with CAR activation in wildtype mice. Overall, the MOA experiments in mice define a very specific PBO CAR MOA as the dominant activator, while simultaneously acknowledging some crosstalk with other liver nuclear receptors. However, primary roles of other nuclear receptor-mediated MOAs for rodent hepatic carcinogens, such as pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR-α), or aromatic hydrocarbon-responsive receptor (AhR) agonism, are ruled out.*

<u>Key Event #2: Increased Cell Proliferation</u>

Induction of RDS in rodent hepatocytes serves as a marker for increased cell proliferation. The thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), is incorporated into DNA during cell proliferation and serves as an immunofluorescent label. In these studies, BrdU-containing osmotic pumps were implanted into the backs of mice to provide a continual source of the nucleotide, then RDS was quantified by the ratio of labeled to total hepatocellular nuclei.

1. Hosako, H. (2015) Piperonyl butoxide – Mode of action (MOA) phase 1: A 14-day dietary study comparing the hepatic effects of piperonyl butoxide and sodium phenobarbital in male CD-1 mice. WIL Research Laboratories, LLC, Ashland, OH. Laboratory Project ID: WIL-782006, May 1, 2015. Unpublished. MRID 51692502.

In a non-guideline, *in vivo* liver tumor MOA study (MRID 51692502), groups of 8-10 CD-1 [Crl:CD-1(ICR)] male mice were administered PBO (9;3.8% a.i.; Lot # E104/10) in the diet at dose levels of 0, 30, 100, or 300 mg/kg/day for 7/8 days with/without a 28-day recovery period or 14 days (cell replication phase), or 14 days with or without a 28-day recovery period (CYP450/peroxisome phase). A positive control, 0.05% NaPB, equivalent to approximately 100 mg/kg/day, was run concurrently. Subcutaneous BrdU pumps were surgically implanted in cell replication assay animals seven days prior to euthanasia. In the cell proliferation assay, 10 animals/dose (including the positive control) were euthanized after exposure for 7, 14, or 8 (plus a 28-day recovery period) days; the latter included control, high-dose, and positive control groups only. The animals underwent routine toxicity examinations including a complete necropsy with selected tissues examined microscopically from animals of the cell replication assay phase only. Also from this phase, whole liver and a section of the duodenum were collected from all animals and processed for BrdU and caspase-3 immunohistochemistry evaluations. The stated purpose of the study was to evaluate the effects of the test substance on hepatocyte proliferation (BrdU) and apoptosis (caspase-3).

The BrdU labeling index (LI) was affected at Day 7 only with statistically significant increases at 300 mg/kg/day (↑6.3-fold) and in the positive control (↑7.2-fold). Treatment at 30 and 100 mg/kg/day resulted in increases (not statistically significant) in BrdU labelling index of ~2-fold. These changes were reversible in the PBO treatment, with some degree of reversibility observed in the positive control (**Table 24**). There were no changes in hepatic apoptosis as determined by caspase-3 LI values after any treatment period or with the positive control (**Table 25**).

Table 24. Mean (± SD) BrdU LI in male mice administered PBO in the diet for 7 or 14 days or 8 days with a 28-day recovery period (cell replication assay groups). [a]

Day	Concentration (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
7	0.640 ± 0.39	1.361 ± 1.70 (↑2.1-fold)	1.418 ± 1.32 (↑2.2-fold)	4.036 ± 2.99** (↑6.3-fold)	4.623 ± 3.66** (↑7.2-fold)
14	1.076 ± 1.21	0.574 ± 0.81	0.581 ± 0.48	0.876 ± 0.71	1.442 ± 0.98
36 [b]	0.301 ± 0.24	-	-	0.228 ± 0.33	0.062 ± 0.08**

a Data were obtained from Text Table 5 on page 52 of MRID 51692502; n = 10.
b 8-day treatment with a 28-day recovery period.
** Significantly different from control; p<0.01.

Table 25. Mean (± SD) caspase-3 LI in male mice administered PBO in the diet for 7 or 14 days or 8 days with a 28-day recovery period (cell replication assay groups). [a]

Day	Concentration (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
7	0.020 ± 0.04	0.030 ± 0.05	0.025 ± 0.05	0.005 ± 0.02	0.0 ± 0.0
14	0.005 ± 0.02	0.0 ± 0.0	0.010 ± 0.02	0.010 ± 0.03	0.010 ± 0.02
36 [b]	0.030 ± 0.04	-	-	0.010 ± 0.02	0.015 ± 0.03

a Data were obtained from Text Table 6 on page 53 of MRID 51692502; n = 10.
b 8-day treatment with a 28-day recovery period.

2. Bowers, M. (2014) Piperonyl butoxide (PBO) – Dietary 7-/14-day study to investigate the hepatic effects of PBO in male constitutive androstane receptor (CAR)/pregnane X receptor (PXR) double knockout and wild type mice. CXR Biosciences, Dundee, Scotland, UK. Laboratory Study No.: CXR1225, May 15, 2014. Unpublished. MRID 51692504.

In a non-guideline, *in vivo* liver tumor MOA study (MRID 51692504), groups of 8 or 10 male C57BL/6J (wild type) and 8 or 10 male PXR KO/CAR KO mice per treatment period were administered PBO (93.9% a.i.; Lot # E104/10) in the diet at target dose levels of 0 and 300 mg/kg/day over 1 week (mean achieved intakes of 0 and 316.4/291.4 mg/kg/day for C57BL/6J mice and 0 and 328.7/235.6 mg/kg/day for PXR KO/CAR KO mice for 7 days/14 days, respectively). Mice were dosed for seven days (Group 1; 10 wild type or KO mice) prior to euthanasia. Osmotic pumps containing BrdU were implanted on Day 1 in mice in the 7-day treatment groups. BrdU labeling indices (LI; for replicative DNA synthesis [S phase]) were evaluated from the liver samples.

After administration of PBO to wild type mice for 7 days, the BrdU LI was increased ($p<0.001$) by 6-fold. After administration of PBO to PXR KO/CAR KO mice for 7 days, the BrdU LI was increased ($p<0.001$) by 4-fold **(Table 26).**

TABLE 26. Mean (± SD) BrdU LI (%) in male mice administered PBO in the diet for 7 days. [a]

Parameter	Dose (mg/kg/day)			
	C57BL/6J		PXR KO/CAR KO	
	0	300	0	300
BrdU LI	1.710 ± 0.679	10.182 ± 1.928*** (↑6-fold)	0.263 ± 0.047	1.060 ± 0.422*** (↑4-fold)

a Data were obtained from Table 6 on page 26 of MRID 51692504; n = 10.
*** Significantly different from control; $p<0.001$.

3. Philips, J.C., Price, R.J., Cunninghame, M.E., et al. (1997) Effect of piperonyl butoxide on cell replication and xenobiotic metabolism in the livers of CD-1 mice and F344 rats. Fund. Appl. Toxicol. 38(1), 64-74. MRID 52376008.

Groups of 8 male CD-1 mice were fed diets containing 0 (control), 10, 30, 100, and 300 mg/kg/day PBO (90.78% a.i.; Lot # not provided) and 0.05% NaPB, the positive control, (w/w; equivalent to 99.4 mg/kg/day) for periods of 7 and 42 days. Replicative DNA synthesis was assessed as the hepatocyte labeling index following implantation of 7-day osmotic pumps containing BrdU during Study Days 0-7 and 35-42.

After 7 days, replicative DNA synthesis, assessed as the hepatocyte Labeling Index, was significantly induced to 348 and 824% of control in mice given 300 mg/kg/day PBO and 0.05% NaPB, respectively. No significant changes in Labeling Index values were observed in mice given 10-100 mg/kg/day PBO for 7 days or any treatment for 42 days (Fig. 8).

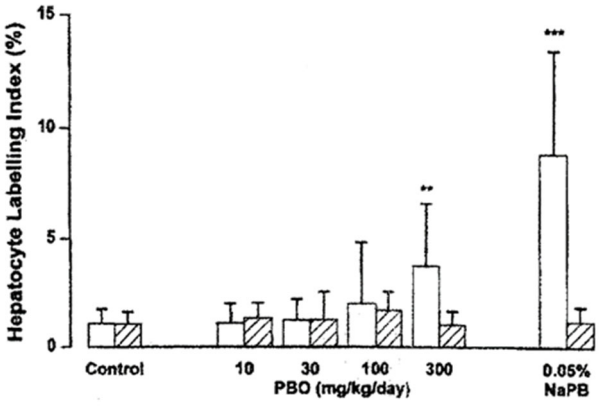

FIG. 3. Effect of dietary administration of 0–300 mg/kg/day PBO and 0.05% NaPB to male CD-1 mice for 7 (open histograms) and 42 (hatched histograms) days on the hepatocyte Labeling Index. Results are means ± SD of eight animals. Values significantly different from control are $^{**}p < 0.01$; $^{***}p < 0.001$.

Figure 8: Hepatocyte Labelling Index

4. Sakamoto, Y., Inoue, K., Takahashi, M., et al. (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. Toxicol. Pathol. 41, pp. 1078-1092. MRID 52376003.

In a carcinogenicity mechanism of action study, groups of male C3H/HeNCrlCrlj (wild-type) or C3H/HeNCrl background constitutive androgen receptor knockout (CAR KO) mice were administered piperonyl butoxide (>90% a.i.; Lot # not provided), decabromodiphenyl ether (DBDE[7]), or sodium phenobarbital (NaPB) in the diet at concentrations of 5000 ppm (~1000 mg/kg/day), 50,000 ppm, or 500 ppm, respectively, for 1, 4, or 27 weeks; groups of 5 animals/treatment/genotype (wild-type or CARKO) were used in the short-term experiments and 20 wild-type or 21-24 CAR KO mice/treatment were used for the 27-week experiment. Experiment #1 was conducted to clarify the role of CAR in mouse liver hypertrophy and induction of metabolism by hepatic enzymes in a 4-week dietary study. Experiment #2 was conducted to investigate the early effects of test item treatment on hepatocyte proliferation in a 1-week dietary study**.**

Replicative DNA synthesis**:** Paraffin-embedded liver sections prepared from animals in Experiments #1 and #2 were evaluated for hepatocyte proliferation. Deparaffinized liver sections were incubated overnight at 4°C with an antibody for proliferating cell nuclear antigen (PCNA, Clone PC10, DAKO). The sections were reacted with secondary antibodies conjugated to peroxidase-labeled dextran polymers (Histofine® Simple Stain mouse MAX PO (R), Nichirei Co., Tokyo, Japan) and visualized by the 3,3'-diaminobenzidine reaction (Sigma Chemical Co., St. Louis, MO), then counterstained with hematoxylin. The percentage of PCNA positive nuclei per a minimum of 1,000 hepatocytes from five randomly selected fields containing both periportal and centrilobular areas was calculated for each animal.

After treatment for one week, the percentages of PCNA-positive hepatocytes were increased (p<0.05) in wild-type animals treated with piperonyl butoxide or NaPB, with a non-significant trend to increase (NS) observed in the piperonyl butoxide-treated CAR KO mice. After treatment for four weeks, labeling

[7] DBDE is a member of a class of brominated flame retardants that are added to plastics.

index values in all groups of both genotypes were <1%. There were no treatment-related differences in labeling indices for piperonyl butoxide-treated mice of either genotype. Labeling indices were decreased (p<0.05) in CAR KO mice treated with DBDE or NaPB, but these changes occurred relative to a control value in CAR KO mice that was increased (p<0.05) compared to the control value in wild-type mice after dietary administration for four weeks. The opposite difference was observed in the control animals after the one-week treatments with a decrease (p<0.05) in labeling index in CARKO mice relative to wild-type mice.

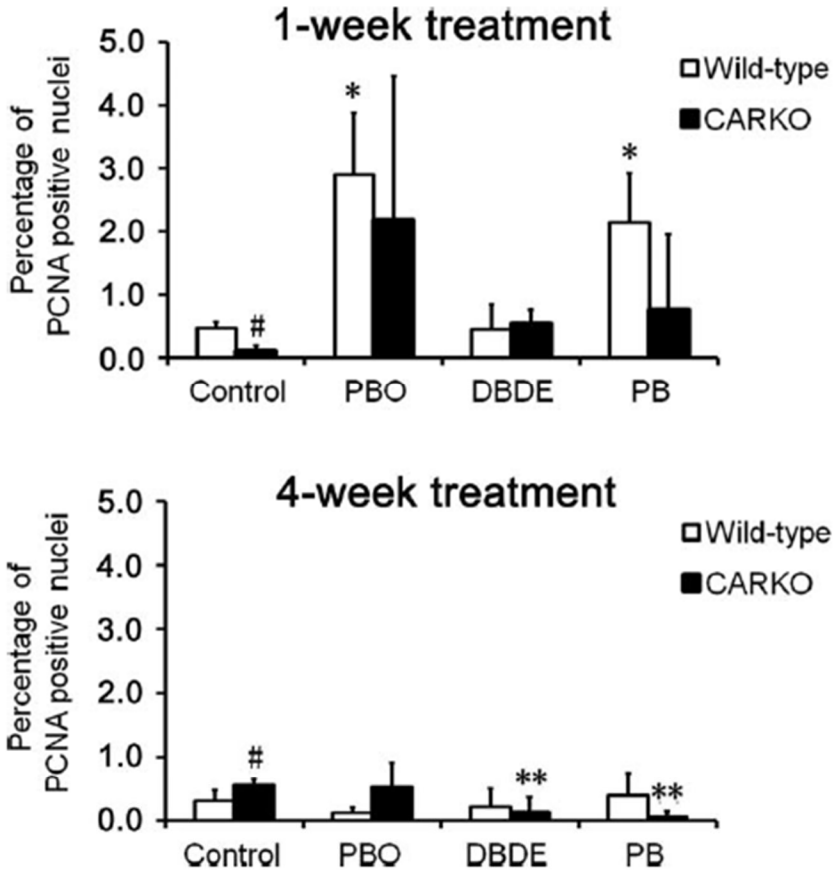

FIGURE 4.—Labeling indices of hepatocytes after the 1- and 4-week treatment of test chemicals. Values represent *Mean* ± *SD* of each group and genotype. * and ** indicate statistically significant differences from the control group of each genotype (*$p < .05$, **$p < .01$: Student and Welch test). # and ## indicate statistically significant differences from the wild-type animals of each group ($^{\#}p < .05$, $^{\#\#}p < .01$: Student and Welch test).

Figure 9: Hepatocyte Labelling Indices

CARC Conclusions for Key Event #2: *The CARC agreed that key event #2 (increased cell proliferation) is adequately supported. A burst of proliferation, measured by a dose-related increase in induction of replicative DNA synthesis was seen at 1 week of PBO treatment of male CD-1 mice treated over 7, 14 and/or 36-42 days at dose levels 30-300 mg/kg/day, including at the tumorigenic dose levels, with statistically significant increases seen only at 300 mg/kg/day. A separate study using wildtype and*

CAR/PXR KO mice administered 300 mg/kg/day PBO for 7 days supported this finding, showing an increase in cell proliferation at 7 days, with a more marked response seen in the wildtype mice than with the double KO mice. A study using wildtype and CAR KO mice given a PBO dose of 1000 mg/kg/day (a dose greater than the tumorigenic dose) for 1 and 4 weeks also supported the initial burst at 7 days only, with the wildtype mice showing a more marked response than the CAR KO mice. These data indicate that at the tumorigenic doses, proliferation is increased in the initial 7 days after dosing before returning to levels similar to or slightly above the baseline proliferation levels, which is the expected proliferation response for CAR-mediated tumorigenesis.

Associative Events #1 and #2 (Increased liver weight and liver hypertrophy):

Activation of CAR was evaluated indirectly following PBO exposure through an increase in liver weight and liver hypertrophy in several mechanistic studies (MRID 51692502, MRID 51692504, MRID 52376008, MRID 52376003) and the cancer mouse bioassay (MRID 42903701).

1. Hosako, H. (2015) Piperonyl butoxide – Mode of action (MOA) phase 1: A 14-day dietary study comparing the hepatic effects of piperonyl butoxide and sodium phenobarbital in male CD-1 mice. WIL Research Laboratories, LLC, Ashland, OH. Laboratory Project ID: WIL-782006, May 1, 2015. Unpublished. MRID 51692502.

In a non-guideline, *in vivo* liver tumor MOA study (MRID 51692502), groups of 8-10 CD-1 [Crl:CD-1(ICR)] male mice were administered PBO (93.8% a.i.; Lot # E104/10) in the diet at concentrations of 0, 30, 100, and 300 mg/kg/day for 7/8 days with/without a 28-day recovery period or 14 days (cell replication phase), or 14 days with or without a 28-day recovery period (CYP450/peroxisome phase). A positive control, 0.05% NaPB, equivalent to approximately 100 mg/kg/day, was run concurrently.

Absolute and relative (to body) liver weights of the cell replication and CYP450 assay groups are presented in **Tables 27** and **Table 28**, respectively. In the cell replication assay groups (**Table 27**), there were no effects of treatment on terminal body weights. In the 7-day treatment groups, there were increases ($p<0.01$) in absolute liver weight at 300 mg/kg/day (↑38%) and relative (to body) liver weights at ≥100 mg/kg/day (↑13% and ↑39%, respectively), as well as absolute and relative (to body) liver weights in the positive control (↑24% and ↑28%, respectively). In the 14-day treatment groups, there were increases ($p<0.01$) in absolute and relative (to body) liver weights at 300 mg/kg/day (↑32% and ↑32%, respectively), as well as relative (to body weight) liver weight in the positive control (↑17%). In the 8-day treatment groups with recovery, there were no effects of treatment on liver weights, including the positive control.

Table 27. Mean (± SD) absolute (g) and relative (to body; %) liver weights in male mice administered PBO in the diet for 7 or 14 days or 8 days with a 28-day recovery period (cell replication assay groups). [a]					
Day	**Concentration (mg/kg/day)**				
	0	**30**	**100**	**300**	**NaPB (0.05%)**
7-day treatment					
Terminal body weight	32.9 ± 1.15	32.7 ± 1.69	32.0 ± 1.59	32.5 ± 1.94	31.8 ± 2.67
Absolute	1.6561 ± 0.08602	1.7990 ± 0.24657	1.8313 ± 0.21374	2.2843 ± 0.12770** (↑38)	2.0565 ± 0.34068** (↑24)
Relative (to body)	5.040 ± 0.0776	5.498 ± 0.6266	5.713 ± 0.4509** (↑13)	7.027 ± 0.2269** (↑39)	6.427 ± 0.6734** (↑28)
8-day treatment (28-day recovery phase)					
Terminal body weight	35.6 ± 2.53	-	-	36.2 ± 2.04	35.3 ± 1.86
Absolute	1.9266 ± 0.21467	-	-	1.9803 ± 0.32607	1.9651 ± 0.18763
Relative (to body)	5.401 ± 0.2961	-	-	5.454 ± 0.6316	5.568 ± 0.3494
14-day treatment					
Terminal body weight	33.8 ± 3.06	33.1 ± 2.56	32.9 ± 2.44	33.9 ± 2.46	33.3 ± 3.73
Absolute	1.8782 ± 0.27441	1.8998 ± 0.29070	1.9873 ± 0.26228	2.4748 ± 0.22200** (↑32)	2.1794 ± 0.42728
Relative (to body)	5.540 ± 0.3232	5.709 ± 0.5230	6.018 ± 0.4528	7.308 ± 0.4692** (↑32)	6.487 ± 0.6305** (↑17)

[a] Data were obtained from Tables S42-S43 on pages 140-141 and Table S45 on page 143 of MRID 51692502; n = 10
Percentage differences from control are presented in parentheses.
** Significantly different from control; p<0.01.

In the CYP450 assay groups (**Table 28**), in the 14-day treatment groups without recovery, there were increases (p<0.05) in absolute liver weight at 300 mg/kg/day (↑29%) and relative (to body) liver weights at ≥100 mg/kg/day (↑8% and ↑29%, respectively), as well as absolute and relative (to body) liver weights in the positive control (↑26% and ↑21%, respectively). There was also a decrease (p<0.05) in relative (to body) liver weight at 30 mg/kg/day (↓9%) but liver weight decrease is not considered toxicologically relevant. In the 14-day treatment group (300 mg/kg/day) with recovery, there were no effects of treatment on liver weights, including the positive control.

TABLE 28. Mean (± SD) absolute (g) and relative (to body; %) liver weights in male mice administered PBO in the diet for 14 days with and without a 28-day recovery period (CYP450 assay groups). [a]

Day	Concentration (mg/kg/day)				
	0	30	100	300	NaPB (0.05%)
14-day treatment (w/o recovery)					
Terminal body weight	32.8 ± 2.07	32.0 ± 1.74	32.0 ± 2.25	32.9 ± 2.80	34.1 ± 1.90
Absolute	1.7288 ± 0.12736	1.5379 ± 0.14738	1.8226 ± 0.19562	2.2295 ± 0.22953** (↑29)	2.1800 ± 0.23671** (↑26)
Relative (to body)	5.270 ± 0.2799	4.801 ± 0.2896* (↓9)	5.694 ± 0.3078* (↑8)	6.782 ± 0.3939** (↑29)	6.391 ± 0.4586** (↑21)
14-day treatment (28-day recovery phase)					
Terminal body weight	35.1 ± 2.41	-	-	35.4 ± 2.76	35.6 ± 1.60
Absolute	1.7241 ± 0.20834	-	-	1.7368 ± 0.24906	1.8388 ± 0.24346
Relative (to body)	4.893 ± 0.3086	-	-	4.895 ± 0.4128	5.162 ± 0.5605

[a] Data were obtained from Table S44 on page 142 and Table S46 on page 144 of MRID 51692502; n = 8. Percentage differences from control are presented in parentheses.

* Significantly different from control; $p<0.05$.

** Significantly different from control; $p<0.01$.

Microscopic pathology findings of the liver in the cell replication assay groups are presented in **Table 29**. In the 300 mg/kg/day animals, there were increased incidences of minimal to mild midzonal hepatocellular hypertrophy in the 7-day, 8-day plus recovery, and 14-day treatment groups (3/10 treated vs. 0/10 control in each phase). In the positive control, there were increased incidences of centrilobular hepatocellular hypertrophy (ranging from minimal to severe) in the 7-day, 8-day plus recovery, and 14-day treatment groups (9-10/10 treated vs. 0/10 control at each phase). All other findings occurred at a similar and smaller incidence in treated groups compared to control, in a single animal, and/or in a manner unrelated to dose.

Table 29. Incidence of selected microscopic findings of the liver in male mice administered PBO in the diet for 7 or 14 days or 8 days with a 28-day recovery period (cell replication assay groups). [a]					
Day	**Concentration (mg/kg/day)**				
	0	**30**	**100**	**300**	**NaPB (0.05%)**
7-day treatment					
Hypertrophy, hepatocellular, centrilobular					
Mild	0	0	0	0	1
Moderate	0	0	0	0	9
Total	0	0	0	0	10
Hypertrophy, hepatocellular, midzonal					
Minimal	0	0	0	2	0
Mild	0	0	0	1	0
Total	0	0	0	3	0
8-day treatment (28-day recovery phase)					
Hypertrophy, hepatocellular, centrilobular					
Minimal	0	0	0	0	3
Mild	0	0	0	0	5
Moderate	0	0	0	0	1
Total	0	0	0	0	9
Hypertrophy, hepatocellular, midzonal					
Minimal	0	0	0	2	0
Mild	0	0	0	1	0
Total	0	0	0	3	0
14-day treatment					
Hypertrophy, hepatocellular, centrilobular					
Mild	0	0	0	0	2
Moderate	0	0	0	0	7
Severe	0	0	0	0	1
Total	0	0	0	0	10
Hypertrophy, hepatocellular, midzonal					
Minimal	0	0	0	2	0
Mild	0	0	0	1	0
Total	0	0	0	3	0

[a] Data were obtained from Tables S47-S49 on pages 145-147 of MRID 51692502; n = 10.

2. Bowers, M. (2014) Piperonyl butoxide (PBO) – Dietary 7-/14-day study to investigate the hepatic effects of PBO in male constitutive androstane receptor (CAR)/pregnane X receptor (PXR) double knockout and wild type mice. CXR Biosciences, Dundee, Scotland, UK. Laboratory Study No.: CXR1225, May 15, 2014. Unpublished. MRID 51692504.

In a non-guideline, *in vivo* liver tumor MOA study (MRID 51692504), groups of 8 or 10 male C57BL/6J (wild type) and 8 or 10 male PXR KO/CAR KO mice per treatment period were administered PBO (93.9% a.i.; Lot # E104/10) in the diet at target dose levels of 0 and 300 mg/kg/day over 1 or two weeks (mean achieved intakes of 0 and 316.4/291.4 mg/kg/day for C57BL/6J mice and 0 and 328.7/235.6 mg/kg/day

for PXR KO/CAR KO mice for 7 days/14 days, respectively). Mice were dosed for either seven days (Group 1; 10 wild type or KO mice) or 14 days (Group 2; 8 wild type of KO mice) prior to euthanasia. At necropsy of the 7-day treatment groups, the liver, gallbladder, and duodenum were excised, the liver was weighed, and tissue samples were obtained from the liver and duodenum. Microscopic pathology was evaluated from the liver samples. At necropsy of the 14-day treatment groups, the liver and gallbladder were excised, and the liver was weighed.

Terminal body weights, and absolute and relative (to body) liver weights of wild type or PXR KO/CAR KO mice treated with PBO for 7 or 14 days are presented in **Table 30**. There were no treatment-related effects on terminal body weights. After treatment for 7 or 14 days in wild type mice, absolute liver weights were increased ($p<0.01$) by 15% and 21%, respectively, and relative (to body) liver weights were increased ($p<0.001$) by 17% and 24%, respectively. After treatment for 14 days in PXR KO/CAR KO mice, relative (to body) liver weight was increased ($p<0.01$) by 9% with no effect on absolute liver weight.

TABLE 30. Mean (± SD) absolute and relative (to body) liver weights in male mice administered PBO in the diet for 7 or 14 days. [a]

Parameter	Dose (mg/kg/day)			
	C57BL/6J		PXR KO/CAR KO	
	0	300	0	300
7-day administration				
Terminal body weight (g)	24.07 ± 1.04	23.61 ± 0.73	25.37 ± 1.66	24.76 ± 1.43
Absolute liver (g)	1.333 ± 0.103	1.531 ± 0.068*** (↑15)	1.448 ± 0.093	1.415 ± 0.208
Relative (to body) liver (%)	5.538 ± 0.395	6.482 ± 0.188*** (↑17)	5.707 ± 0.133	5.700 ± 0.702
14-day administration				
Terminal body weight (g)	24.06 ± 1.43	23.68 ± 0.91	25.90 ± 1.82	25.08 ± 1.50
Absolute liver (g)	1.273 ± 0.193	1.544 ± 0.093** (↑21)	1.393 ± 0.148	1.473 ± 0.141
Relative (to body) liver (%)	5.275 ± 0.594	6.522 ± 0.301*** (↑24)	5.369 ± 0.254	5.868 ± 0.355** (↑9)

a Data were obtained from Table 4 on page 24 of MRID 51692504; n = 10 for the 7-day. exposures and n = 8 for the 14-day exposures.
** Significantly different from control; $p<0.01$.
*** Significantly different from control; $p<0.001$.

In the wild type mice, slight centrilobular hypertrophy (10/10 treated vs. 0/10 control) was seen at 300 mg/kg/day (**Table 31**). In the PXR KO/CAR KO mice, there was no evidence of hepatocellular hypertrophy in treated PXR KO/CAR KO mice. Vacuolation and mitotic figures did not differ from the control for both the wild type and KO mice.

TABLE 31. Incidence of selected microscopic findings in the liver of male mice administered PBO in the diet for 7 days. [a]

Finding		Concentration (ppm)			
		C57BL/6J		PXR KO/CAR KO	
		0	300	0	300
Number of mice examined		**10**	**10**	**10**	**10**
Vacuolation	Total	10	9	9	7
	Minimal (grade 1)	-	3	2	3
	Slight (grade 2)	5	6	2	3
	Moderate (grade 3)	5	-	5	1
Mean grade/tissue examined		2.5	1.5	2.1	1.2
Hepatocellular hypertrophy	Total	-	10	-	-
	Slight	-	10	-	-
Mitotic figures	Total	7	9	4	3
	Minimal	7	6	4	2
	Slight	-	3	-	1

A Data were obtained from Table 7 on pages 27-28 of MRID 51692504.
- Not present.

3. Philips, J.C., Price, R.J., Cunninghame, M.E., et al. (1997) Effect of piperonyl butoxide on cell replication and xenobiotic metabolism in the livers of CD-1 mice and F344 rats. Fund. Appl. Toxicol. 38(1), 64-74. MRID 52376008.

Groups of 8 male CD-1 mice were fed diets containing 0 (control), 10, 30, 100, or 300 mg/kg/day PBO (90.78% a.i.; Lot # not provided) and 0.05% NaPB, the positive control, (w/w; equivalent to 99.4 mg/kg/day) for periods of 7 and 42 days. The liver and small intestines were excised from each animal, and liver weights were determined. Portions of each liver were collected and prepared for microscopic evaluation and for evaluation of liver homogenates and slices.

The administration of 300 mg/kg/day PBO for 7 days, 100 or 300 mg/kg/day PBO for 42 days, and 0.05% NaPB for 7 and 42 days significantly increased relative liver weight. No tabular data were presented in the article. See Figure 10 below.

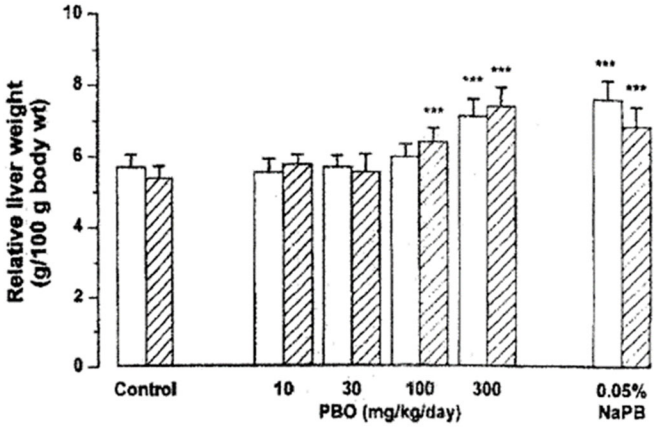

FIG. 1. Effect of dietary administration of 0–300 mg/kg/day PBO and 0.05% NaPB to male CD-1 mice for 7 (open histograms) and 42 (hatched histograms) days on relative liver weight. Results are means ± SD of eight animals. Values significantly different from control are ***$p < 0.001$.

Figure 10: Relative Liver Weights

Microscopic changes attributed to treatment with PBO consisted of midzonal hepatocyte hypertrophy at 100 mg/kg/day (42-day treatment) and 300 mg/kg/day (7- and 42-day treatments). Centrilobular hepatocyte hypertrophy was observed in the NaPB-treated animals after both treatment periods. (No incidence data were available. This information was from qualitative text in the article).

4. Hermansky, S.J. and Wagner, C.L. (1993) Chronic dietary oncogenicity study with piperonyl butoxide in CD-1® mice. Bushy Run Research Center, Union Carbide Chemicals and Plastics Company, Inc., Export, PA. Laboratory Study ID: 91N0134, August 27, 1993. Unpublished. MRID 42903701.

In a carcinogenicity study (MRID 42903701*)*, groups of 60 CD-1® mice/sex were administered PBO (90.78% a.i.; Lot # FEP-100 12/12/89) via the diet at dose levels of 0 (Group 1), 0 (Group 2), 30, 100, or 300 mg/kg/day for up to 78 weeks.

In the liver, dose-dependent treatment-related increases (at $p<0.01$ or $p<0.05$) were observed in absolute liver weight/relative (to body) liver weight /relative (to brain) liver weight, respectively, at 100 mg/kg/day in males (↑19%/↑16%/↑20%, respectively) and in females (↑11%/↑8%/↑14%, respectively) and at 300 mg/kg/day in males (↑67%/↑67%/↑70, respectively) and in females(↑20%/↑19%/↑20%, respectively). There were no treatment-related effects on absolute or relative liver weights at 30 mg/kg/day or any other organ weights at any dose.

Table 32. Absolute (g), relative (to body, %), and relative (to brain, %) liver weights in mice administered PBO in the diet for up to 78 weeks. [a]				
Observation	**Dose (mg/kg/day)**			
	0 (Group 1)	**30**	**100**	**300**
Males				
Term. BW (g)	42.8 ± 4.64	43.6 ± 5.44	43.2 ± 4.44	41.9 ± 3.88
Abs. Liver (g)	2.560 ± 0.641	2.817 ± 1.178	3.037 ± 1.244* (↑19)	4.281 ± 2.000** (↑67)
Rel. (to body) Liver (%)	6.060 ± 1.731	6.492 ± 2.593	7.038 ± 2.823 (↑16)	10.130 ± 4.309** (↑67)
Rel. (to brain) Liver (%)	491.174 ± 132.286	544.664 ± 223.582	591.546 ± 250.279* (↑20)	836.087 ± 393.344** (↑70)
Females				
Term. BW (g)	38.1 ± 4.87	37.6 ± 4.20	38.9 ± 4.20	38.1 ± 4.71
Abs. Liver (g)	2.208 ± 0.496	2.182 ± 0.625	2.446 ± 0.430* (↑11)	2.657 ± 0.505** (↑20)
Rel. (to body) Liver (%)	5.822 ± 1.249	5.779 ± 1.296	6.308 ± 1.023 (↑8)	6.955 ± 0.759** (↑19)
Rel. (to brain) Liver (%)	420.847 ± 96.225	414.716 ± 129.636	477.951 ± 85.117* (↑14)	506.859 ± 97.712** (↑20)

a Data obtained from Appendix 3, Tables 1-6 on pages 131-136 of MRID 42903701; n = 36-47 in males and 37-49 in females. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.

* Significantly different from control; p<0.05.

** Significantly different from control; p<0.01.

Selected macroscopic pathology findings are presented in **Table 33.** Treatment-related macroscopic pathology findings were observed in the livers of both sexes. They consisted of increased incidences of masses and nodules (combined) at ≥100 mg/kg/day in males (28-37/60 treated vs. 9-18/60 controls) and at 300 mg/kg/day in females (8/60 treated vs. 2/60 each control), increased size in the 300 mg/kg/day males (6/60 treated vs. 0-1 controls), and focal/multifocal color change in the 300 mg/kg/day males (13/60 treated vs. 4-8/60 controls). It was noted that not all hepatic nodules/masses were confirmed as hepatocellular neoplasms during microscopic evaluation. The number of animals with observed neoplasms during macroscopic evaluation was less than the combined incidence of nodules and masses.

Table 33. Selected macroscopic findings in the livers of mice administered PBO in the diet for up to 78 weeks. [a]										
Organ	**Sex and dose (mg/kg/day)**									
	Males					**Females**				
	0 (Group 1)	**0 (Group 2)**	**30**	**100**	**300**	**0 (Group 1)**	**0 (Group 2)**	**30**	**100**	**300**
# of animals examined	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**
Liver										
Nodule	10	2	1	9	9	1	2	1	1	2
Mass	8	7	12	19	28	1	0	1	1	6
Size increase	0	1	0	0	6	1	0	0	1	1
Color change, focal/multifocal	4	8	5	5	13	9	6	7	7	8

a Data were obtained from Appendix 3, Tables 9 and 12 on pages 146-147, and 163 of MRID 42903701.

Selected non-neoplastic microscopic pathology findings are presented in **Table 34**. An increased incidence (p<0.05) of hepatocellular hypertrophy was observed in the 100 mg/kg/day males (16/60 treated vs. 6/60 Group 1 control only). At 300 mg/kg/day, treatment-related non-neoplastic

microscopic findings included increased (p≤0.01) incidences of hepatocellular hypertrophy in males (43/60 treated vs. 6-11/60 controls) and females (9/60 treated vs. 0-4/60 controls), eosinophilic foci in males (5/60 treated vs 0-1/60 controls) and in female (4/60 treated vs 0/60 controls), and hemorrhage in males (13/60 treated vs. 1-2/60 controls) and females (7/60 (NS) vs 3/60 (both controls). There was also an increased incidence (NS) of hyperplasia at the high dose in both sexes.

Table 34. Selected non-neoplastic microscopic pathology findings in mice after administration of piperonyl butoxide in the diet for up to 78 weeks. [a]

	Dose (mg/kg/day)									
	Males					Females				
Tissue	0 (Group 1)	0 (Group 2)	30	100	300	0 (Group 1)	0 (Group 2)	30	100	300
# of animals examined	60	60	60	60	60	60	60	60	60	60
Liver										
Hepatocellular hypertrophy	6	11	11	16*	43** §§	0	4	0	1	9**
Eosinophilic foci[b]	1	0	0	0	5	0	0	0	1	4
Hemorrhage	2	1	3	2	13** §§	3	3	1	4	7
Hepatocellular Hyperplasia	0	2	1	2	5	0	0	0	1	4

a Data were obtained from Appendix 3, Tables 15 and 19 on pages 194 and 241-242 of MRID 42903701.
b Data were obtained from registrant's MOA submission (MRID 51692501).
* Significantly different from Group 1 control; p≤0.05.
** Significantly different from Group 1 control; p≤0.01.
§§ Significantly different from Group 2 control; p≤0.01.

5. Sakamoto, Y., Inoue, K., Takahashi, M., et al. (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. Toxicol. Pathol. 41, pp. 1078-1092. MRID 52376003.

In a carcinogenicity mechanism of action study, groups of male C3H/HeNCrlCrlj (wild-type) or C3H/HeNCrl background CARKO mice were administered piperonyl butoxide (>90% a.i.; Lot # not provided), decabromodiphenyl ether (DBDE[8]), or sodium phenobarbital (NaPB) in the diet at concentrations of 5000 ppm (~1000 mg/kg/day), 50,000 ppm, or 500 ppm, respectively, for 1, 4, or 27 weeks; groups of 5 animals/treatment/genotype (wild-type or CARKO) were used in the short-term experiments and 20 wild-type or 21-24 CARKO mice/treatment were used for the 27-week experiment. Experiment #1 was conducted to clarify the role of CAR in mouse liver hypertrophy and induction of metabolism by hepatic enzymes in a 4-week dietary study. Experiment #2 was conducted to investigate the early effects of test item treatment on hepatocyte proliferation in a 1-week dietary study. Experiment #3 was carried out to investigate the role of CAR in mouse hepatocarcinogenesis pathways by using a standard protocol for tumor initiation and promotion (Diwan *et al.*, 1986[9]). Mice were administered a single intraperitoneal injection of 90 mg/kg diethylnitrosamine (DEN) one week prior to the initiation of the 27-week study. Body weights and food intake were monitored throughout

[8] DBDE is a member of a class of brominated flame retardants that are added to plastics.

[9] Diwan, B.A., Rice, J.M., Ohshima, M., *and Ward, J.M.* (1986) Interstrain differences in susceptibility to liver carcinogenesis initiated by N-nitrodiethylamine and its promotion by Phenobarbital in C57BL/6NCr, C3H/HeNCrMTV-, and DBA/2NrCr mice. *Carcinogenesis* 7, 215-20.

treatment. At each termination time, the livers were excised and weighed. Liver sections from the 4-week study were examined microscopically and stained for Cyp2b synthetic peptide and liver sections from the short-term experiments were evaluated for hepatocyte proliferation. Liver samples from the 4-week study were also evaluated for treatment-related changes in mRNA expression by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Livers from the 27-week experiment were evaluated macroscopically for nodule formation and evaluated microscopically for treatment-related hepatic lesions such as adenomas and foci of hepatocellular alteration.

Liver weight: Liver weight data for all animals in Experiments #1, #2, and #3 are presented in **Table 35**. Absolute and relative (to body) liver weights were increased (p<0.01) by 50-100% and 47-135%, respectively, in PBO-treated animals of both genotypes and increased (p<0.01) by 21-40% and 26-44%, respectively, in NaPB-treated wild-type mice only. Absolute and relative (to body) liver weights were increased (p<0.01) by 27-29% and 27-28%, respectively, in DBDE-treated wild-type animals after administration for 4 and 27 weeks, with relative (to body) liver weight increased (p<0.01) by 11% in CARKO mice only after administration for 27 weeks. In CARKO mice administered NaPB, absolute liver weight was decreased (p<0.01) by 19% after 27 weeks and relative (to body) liver weights were decreased (p<0.05) by 4-11% in the 4- and 27-week treatment groups.

Table 35. Mean (± SD) absolute and relative (to body) liver weights (g and %, respectively) in male mice administered piperonyl butoxide, DBDE, or NaPB in the diet for 1, 4, or 27 weeks. [a]

Test group	Diet concentration (ppm)	Experiment/Treatment Period		
		#1/4 weeks	#2/1 week	#3/27 weeks
Absolute liver weight (Wild-type mice)				
Control	0	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.2 [b]
Piperonyl butoxide	5000[f]	2.2 ± 0.1** (↑57)	2.1 ± 0.1** (↑50)	3.0 ± 0.4** (↑100) [b]
DBDE	50,000	1.8 ± 0.2** (↑29)	1.4 ± 0.1	1.9 ± 0.2** (↑27) [b]
NaPB	500	1.7 ± 0.1** (↑21)	1.8 ± 0.2** (↑29)	2.1 ± 0.4** (↑40) [b]
Absolute liver weight (CARKO mice) [c]				
Control	0	1.6 ± 0.2	1.2 ± 0.2	1.6 ± 0.2 [d]
Piperonyl butoxide	5000	2.6 ± 0.2** (↑63)	1.8 ± 0.2** (↑50) [e]	2.4 ± 0.2** (↑50) [d]
DBDE	50,000	1.6 ± 0.1	1.4 ± 0.1	1.8 ± 0.3 [d]
NaPB	500	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1** (↓19) [d]
Relative (to body) liver weight (Wild-type mice)				
Control	0	4.9 ± 0.2	5.8 ± 0.1	4.3 ± 0.5 [b]
Piperonyl butoxide	5000	8.6 ± 0.2** (↑76)	9.1 ± 0.6** (↑57)	10.1 ± 1.3** (↑135) [b]
DBDE	50,000	6.2 ± 0.2** (↑27)	5.6 ± 0.2	5.5 ± 0.3** (↑28) [b]
NaPB	500	6.2 ± 0.1** (↑27)	7.3 ± 0.4** (↑26)	6.2 ± 1.0** (↑44) [b]
Relative (to body) liver weight (CARKO mice) [c]				
Control	0	5.3 ± 0.3	5.5 ± 0.2	4.6 ± 0.3 [d]
Piperonyl butoxide	5000	9.1 ± 0.8** (↑72)	8.1 ± 0.7** (↑47) [e]	7.8 ± 0.8** (↑70) [d]
DBDE	50,000	5.3 ± 0.1	5.7 ± 0.2	5.1 ± 0.5** (↑11) [d]
NaPB	500	4.7 ± 0.1** (↓11)	6.0 ± 0.2	4.4 ± 0.2* (↓4) [d]

a Data were obtained from Table 1 on page 1080 of Sakamoto *et al.* (2013); n = 5/treatment group/genotype, except as noted otherwise. Percentage differences from control (calculated by the Reviewers) are presented in parentheses.
b n = 20.
c Constitutive androgen receptor (CAR) knockout.
d n = 21-24.
e n = 4.
f 5000 ppm corresponds to ~1000 mg/kg/day.
* Significantly different from control; $p < 0.05$.
** Significantly different from control; $p < 0.01$.

Liver Hypertrophy: In wild-type mice treated for 4 weeks, marked hepatocellular hypertrophy was observed in PBO-treated animals, with moderate and moderate to marked centrilobular hypertrophy in NaPB-treated animals. In the CARKO mice, mild to moderate hepatocellular hypertrophy was observed in PBO-treated animals. There were no incidences of hepatocellular hypertrophy in the NaPB-treated CARKO mice. See **Table 36.**

Table 36. Microscopic non-neoplastic pathology findings in the livers of wild-type (Wild) and CARKO (KO) mice treated for four weeks (Experiment #1).

	Control		PBO		DBDE		PB	
Findings	Wild $N = 5$	KO $N = 5$	Wild $N = 5$	KO $N = 4$	Wild $N = 5$	KO $N = 5$	Wild $N = 5$	KO $N = 5$
Hepatocellular hypertrophy								
Mild	0	0	0	2[a]	0	0	0	0
Moderate	0	0	0	2[b]	5[a]	0	3[a]	0
Marked	0	0	5[b]	0	0	0	2[a]	0
Neutrophil infiltration								
Mild	0	0	1	2	0	0	0	0
Focal necrosis								
Mild	0	0	0	1	0	0	0	0
Vacuolation, hepatocyte[c]								
Mild	0	0	0	3	0	2	0	0

Note. CARKO = CAR knockout; PBO = piperonyl butoxide; DBDE = decabromodiphenyl ether; PB = phenobarbital.
"Wild" indicates wild-type mice. "KO" indicates CARKO mice.
The numbers in column indicate the numbers of animals that showed each finding.
[a]Hepatocellular hypertrophy was found at centrilobular area.
[b]Hepatocellular hypertrophy was found at centrilobular to midzonal area.
[c]Vacuolation of hepatocytes was found in centrilobular area.

Liver Hypertrophy: In wild-type mice treated for 27 weeks, moderate (n=5) to marked (n=12) hepatocellular hypertrophy was observed in PBO-treated animals, with marked (n=18) centrilobular hypertrophy observed in NaPB-treated animals. In the CARKO mice, mild (n=2), moderate (n=11) and marked (n=7) hepatocellular hypertrophy was observed in PBO-treated animals. There were no incidences of hepatocellular hypertrophy in the NaPB-treated CARKO mice. **See Table 37.**

Table 37. Microscopic non-neoplastic pathology findings in the livers of wild-type (Wild) and CARKO (KO) mice treated for 27 weeks (Experiment #3).

	Control		PBO		DBDE		PB	
Findings	Wild $N = 20$	KO $N = 20$	Wild $N = 17$	KO $N = 20$	Wild $N = 18$	KO $N = 20$	Wild $N = 18$	KO $N = 19$
Hepatocellular hypertrophy[a]								
Mild	0	0	0	2	1	11	0	0
Moderate	0	0	5	11	16	7	0	0
Marked	0	0	12	7	1	0	18	0
Karyocytomegaly								
Mild	0	0	6	5	6	7	9	0
Moderate	0	0	2	0	0	1	9	0
Neutrophil infiltration								
Mild	0	0	1	0	0	9	0	0
Focal necrosis								
Mild	0	0	3	7	1	1	0	0
Moderate	0	0	0	1	0	0	0	0
Single cell necrosis								
Mild	0	0	0	0	2	0	0	0
Yellow pigment deposition[b]								
Mild	0	0	1	1	8	3	4	1
Vacuolation, centrilobular[c]								
Mild	0	0	0	5	0	5	0	0
Moderate	0	0	0	3	0	3	0	0

Note. CARKO = CAR knockout; PBO = piperonyl butoxide; DBDE = decabromodiphenyl ether; PB = phenobarbital.
"Wild" indicates wild-type mice. "KO" indicates CARKO mice.
The numbers in column indicate the numbers of animals which showed each finding.
[a]Hepatocellular hypertrophy was observed in centrilobular area.
[b]Yellowish pigment deposition was found in foci of histiocytes and this change was occasionally accompanied by mononuclear cell infiltration and oval cell hyperplasia around periportal area.
[c]Vacuolations in centrilobular area were supposed to be lipid accumulations which were seen in the same groups of CARKO mice in Exp. 3.

CARC Conclusions on Associative Events #1 (increased liver weight) and #2 (increased liver hypertrophy): *The CARC agreed that Associative Events #1 and #2 are adequately supported by the data. Activation of CAR was evaluated indirectly following PBO exposure through an increase in liver weight and liver hypertrophy.*

At 300 mg/kg/day, robust statistically significant increases were seen in liver weight after 7, 14, and 42 days exposure to PBO in CD-1 male mice as well as male C57BL/6J wildtype mice, while no effect on liver weight was seen in male PXR KO/CAR KO mice after 7 or 14 days. No changes in absolute liver weights were seen at 100 mg/kg/day for the shorter time points, but a statistical increase in relative liver weight was seen at 7 and 42 days. Dose related statistically significant increased liver weights were seen following 78 weeks exposure to PBO at 100 and 300 mg/kg/day in both male and female CD-1 mice. The increased liver weights seen at 1000 mg/kg/day PBO in both male C3H/HeN/CRlCrjl wildtype and CAR KO mice treated for 1, 4 and 27 weeks, may be the result of crosstalk with the CAR and PXR receptors.

Increased liver hypertrophy was seen at 7 and 14 days at 300 mg/kg/day PBO in male CD-1 mice and male C57BL/6J wildtype mice. There was no evidence of liver hypertrophy seen in the C57BL/6J PXR KO/CAR KO male mice at 300 mg/kg/day at these early timepoints. Increased liver hypertrophy was observed in CD-1 males at ≥100 mg/kg/day and in CD-1 females at 300 mg/kg/day following 78-weeks exposure to PBO. Some evidence of cross talk between CAR and PXR receptors was seen in a study where both male CH3/HeNCrlCrlj wildtype and CH3/HeNCrlCrlj CAR KO mice showed increased liver hypertrophy at 4 and 27 weeks following administration of a high dose of PBO (1000 mg/kg/day), but with the difference being that the wildtype mice showed a greater severity of liver hypertrophy than the CAR KO mice.

<u>Key Event #3: Clonal Expansion Leading to Altered Hepatic Foci</u>

1. Hermansky, S.J. and Wagner, C.L. (1993) Chronic dietary oncogenicity study with piperonyl butoxide in CD-1® mice. Bushy Run Research Center, Union Carbide Chemicals and Plastics Company, Inc., Export, PA. Laboratory Study ID: 91N0134, August 27, 1993. Unpublished.
MRID 42903701.

Treatment of male and female mice with PBO resulted in a small increase in the incidence of eosinophilic foci in males at 300 mg/kg/day and in females at ≥100 mg/kg/day at 78 weeks (see **Table 34** above).

2. Sakamoto, Y., Inoue, K., Takahashi, M., et al. (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. Toxicol. Pathol. 41, pp. 1078-1092. MRID 52376003.

In a carcinogenicity mechanism of action study, groups of male C3H/HeNCrlCrlj (wild-type) or C3H/HeNCrl background CARKO mice were administered PBO (>90% a.i.; Lot # not provided), decabromodiphenyl ether (DBDE[10]), or NaPB in the diet at concentrations of 5000 ppm (~1000 mg/kg/day), 50,000 ppm, or 500 ppm, respectively, for 1, 4, or 27 weeks; groups of 5

[10] DBDE is a member of a class of brominated flame retardants that are added to plastics.

animals/treatment/genotype (wild-type or CARKO) were used in the short-term experiments and 20 wild-type or 21-24 CARKO mice/treatment were used for the 27-week experiment. Body weights and food intake were monitored throughout treatment. This experiment was carried out to investigate the role of CAR in mouse hepatocarcinogenesis pathways by using a standard protocol for tumor initiation and promotion. Mice were administered a single intraperitoneal injection of 90 mg/kg diethylnitrosamine (DEN[11]) one week prior to the initiation of the 27-week study. Livers from the 27-week experiment were evaluated macroscopically for nodule formation and evaluated microscopically for treatment-related hepatic lesions such as adenomas and foci of hepatocellular alteration.

Treatment-related effects on proliferative hepatic lesions included increased incidences of eosinophilic foci in all PBO and NaPB-treated wild-type mice, with fewer or no incidences in CARKO mice at 27 weeks (**Table 38**). There were generally no treatment-related effects on the incidences of basophilic foci, except decreases in both parameters in NaPB-treated CARKO mice. There were generally no treatment-related effects on other types of foci in PBO-treated animals.

Table 38. Incidences of proliferative hepatic lesions in wild-type (Wild) and CARKO (KO) mice treated for 27 weeks after DEN initiation

		Control		PBO		DBDE		PB	
Neoplastic lesion		Wild $N = 20$	KO $N = 20$	Wild $N = 17$	KO $N = 20$	Wild $N = 18$	KO $N = 20$	Wild $N = 18$	KO $N = 19$
Eosinophilic	Altered foci	5	9	17**	13$^{\#}$	9	1*$^{\#\#}$	18**	0*$^{\#\#}$
	Adenomas	0	2	17**	7$^{\#\#}$	2	0	18**	0$^{\#\#}$
Basophilic	Altered foci	18	13	16	14	18	18	15	5*$^{\#\#}$
	Adenomas	19	10$^{\#\#}$	16	15	17	18*	13	1**$^{\#\#}$
Other type	Altered foci	8	8	6	4	16**	4$^{\#\#}$	15*	0**$^{\#\#}$
	Adenomas	2	1	7	3	6	1	13**	0$^{\#\#}$

Note. CARKO = CAR knockout; PBO = piperonyl butoxide; DBDE = decabromodiphenyl ether; PB = phenobarbital.
"Wild" indicates wild-type mice. "KO" indicates CARKO mice.
The numbers in column indicate the numbers of animals in which one or more lesions were found.
Significantly different from the control group of each genotype: (*$p < .05$, **$p < .01$: Fisher's test).
Significantly different from wild-type mice of each group: ($^{\#}p < .05$, $^{\#\#}p < .01$: Fisher's test).

CARC Conclusions on Key Event #3 (Clonal Expansion Leading to Altered Hepatic Foci): *The CARC considered this key event to be adequately supported at 300 mg/kg/day. Treatment of male and female CD-1 mice with 300 mg/kg/day PBO resulted in a small increase in the incidence of eosinophilic foci at 78 weeks, but no evidence of foci was seen in males at 100 mg/kg/day, the tumorigenic dose in this sex. In addition, there was a slight increase in liver hyperplasia seen in both sexes at 300 mg/kg/day. Statistically increased altered eosinophilic foci were also seen at an earlier time point (27 weeks) in a study with male C3H/HeNCrlCrlj (wild-type) mice treated with a high dose of PBO (1000 mg/kg/day). Fewer foci were seen in CAR KO mice in this study, which may be due to crosstalk between CAR and PXR receptors.*

Key Event #4 (liver tumor formation):

Hermansky, S.J. and Wagner, C.L. (1993) Chronic dietary oncogenicity study with piperonyl butoxide in CD-1® mice. Bushy Run Research Center, Union Carbide Chemicals and Plastics Company, Inc., Export,

[11] N-Diethylnitrosamine (DEN) is known as both a hepatotoxin and hepatocarcinogen. It is a substrate of CYP2E1 and plays an active role in reactive oxygen species (ROS) generation.

PA. Laboratory Study ID: 91N0134, August 27, 1993. Unpublished.
MRID 42903701.

The final key event is the formation of liver tumors in male and female mice. As described in Section IV.A.3, a statistically significant increase in combined liver adenomas and/or carcinomas occurred in males at ≥100 mg/kg/day and in adenomas only in females at 300 mg/kg/day in a 78-week dietary study in mice.

CARC Conclusions on Key Event #4 (liver tumor formation): *The CARC determined that liver tumors, driven by adenomas, were treatment related at ≥100 mg/kg/day in males and 300 mg/kg/day in females in a 78-week dietary study in mice.*

B. Dose and Temporal Concordance

Extracted from the Registrant's MOA white paper (MRID 51692501):

> *Dose-Response Concordance:*
> *A summary of the dose-concordance for the key and associative events for PBO-induced liver tumor formation in male CD-1 mice is shown in Table 36. While liver tumor formation was not increased in male CD-1 mice given 30 mg/kg/day PBO, treatment with 100 and 300 mg/kg/day PBO resulted in statistically significant increases in the incidence of hepatocellular adenoma and in the combined incidence of hepatocellular adenoma and carcinoma. The key events of hepatic CAR activation (inferred from induction of hepatic Cyp2b enzyme activity and mRNA levels), increased hepatocyte RDS, and liver tumor formation and the associative events of liver weight and hypertrophy (increased liver weight hepatocyte hypertrophy) were observed in male CD-1 mice given the carcinogenic dose levels of 100 and 300 mg/kg/day PBO and were dose-dependent. Due to inter-animal variation, the treatment of male CD-1 mice with 100 mg/kg/day PBO did not result in a statistically significant increase in hepatocyte RDS. However, the >2 fold increase in hepatocyte RDS is considered to be biologically significant and is in agreement with a previous study (Phillips et al., 1997). As shown in Table 5a, the treatment of male CD-1 mice with both 100 and 300 mg/kg/day PBO did not result in a very marked increase in the incidence of hepatocellular adenoma compared to the observed incidence in control male CD-1 mice, thus demonstrating a variation in response between different animals. In addition to the effects observed in male CD-1 mice given carcinogenic doses of 100 and 300 mg/kg/day PBO, significant increases in the key event of hepatic CAR activation, as indicated by the associative event of induction of Cyp2b enzymes, was also observed at the noncarcinogenic dose level of 30 mg/kg/day PBO. For the key event of altered hepatic foci, a small increase was only observed in male mice given 300 mg/kg/day PBO, but as described above this is not considered to detract from the proposed MOA. Overall, there are strong parallels between the dose-response relationships for the key and associative events and the subsequent liver tumor response.*
>
> *As shown in Table 39, the hepatic effects of PBO in CD-1 mice are very similar to the key and associative events previously reported for phenobarbital and a number of other*

nongenotoxic CAR activators which produce liver tumors in the mouse and/or rat (Cohen, 2010; Elcombe et al., 2014; Lake, 2018; La Rocca et al., 2017; LeBaron et al., 2014; Okuda et al., 2017a; Osimitz and Lake, 2009; Peffer et al., 2018; Tinwell et al., 2014; Wiemann, et al., 2019; Yamada, 2018; Yamada et al., 2009, 2021).

Table 39. Dose-dependent effects of PBO on key and associative events for male CD-1 mouse liver tumor formation (excerpted from MRID 51692501)

Key (KE) or associative (AE) event	Male CD-1 mice PBO (mg/kg/day)		
	30	100	300
CAR activation (KE)[a]	Yes	Yes	Yes
Increased relative liver weight (AE)[b]	No	Yes	Yes
Hepatocyte hypertrophy (AE)[c]	No	Yes	Yes
Increased hepatocyte RDS (KE)[d]	Yes	Yes	Yes
Induction of Cyp2b enzymes (AE)[e]	Yes	Yes	Yes
Formation of altered liver foci (KE)[f]	No	No	Yes
Liver tumours (KE)	No	Yes	Yes

[a] Inferred from induction of microsomal PROD activity and hepatic Cyp2b10 mRNA levels at all PBO dose levels in CD-1 mice and induction of microsomal Cyp2b protein levels at PBO dose levels of 100 and 300 mg/kg/day. Activation of CAR was also demonstrated in the studies with C57BL/6J (wild type) and CAR KO/PXR KO mice. Treatment with PBO markedly induced hepatic Cyp2b enzyme activity and Cyp2b10 mRNA levels in C57BL/6J mice, whereas in CAR KO/PXR KO mice PBO treatment only produced a small increase in hepatic Cyp2b enzyme activity and a significant decrease in Cyp2b10 mRNA levels (Lake, 2013).

[b] Increased relative liver weight was observed after 7, 14 and 42 days and after 79 weeks of treatment (Butler et al., 1998; Hosako, 2015; Phillips et al., 1997).

[c] Hepatocyte hypertrophy (determined by morphological examination of liver sections) was observed in mice given 300 mg/kg/day PBO for 7, 14 and 42 days and after 79 weeks of treatment and in mice given 100 mg/kg/day PBO for 42 days and after 79 weeks of treatment (Butler et al., 1998; Hosako, 2015; Phillips et al., 1997).

[d] Hepatocyte replicative DNA synthesis (RDS) was significantly increased in two studies in mice given 300 mg/kg/day PBO for 7 days (Lake et al., 2020; Phillips et al., 1997). The treatment of CD-1 mice with 30 and 100 mg/kg/day PBO for 7 days in two studies resulted in increases in hepatocyte RDS to around 200% of control at both doses (Hosako, 2015) and 186% of control at 100 mg/kg/day (Phillips et al., 1997). However, owing to inter-animal variation these increases were not statistically significant, but are considered to be biologically significant.

[e] The treatment of mice with 30-300 mg/kg/day PBO for 14 days resulted in significant increases in hepatic microsomal Cyp2b enzyme (PROD) activity and hepatic Cyp2b10 mRNA levels, with induction of microsomal Cyp2b protein levels also being observed at PBO doses of 100 and 300 mg/kg/day (Lake, 2012).

[f] A small increase in eosinophilic foci was observed in male mice treated with 300 mg/kg/day PBO for 79 weeks

Extracted from the Registrant's MOA white paper (MRID 51692501):

Temporal Association:
If a key event (or events) is an essential element for hepatocarcinogenesis it must occur before the appearance of liver tumors. Thus it is critical in the evaluation of a MOA for rodent liver tumor formation that effects on the key and associative events occur before the appearance of liver tumors. Data are available for the hepatic effects of PBO treatment in male CD-1 mice (the bioassay strain) at a number of time points including 7, 14 and 42 days and 79 weeks of treatment. Short-term treatment of male CD-1 mice resulted in significant increases in hepatic Cyp2b subfamily enzyme activity and mRNA

levels. Treatment with PBO also resulted in significant increases in relative liver weight, which was associated with hepatocyte hypertrophy, at early time points (e.g. 7, 14, and 42 days) and also after 79 weeks of PBO administration.

Although PBO produced the greatest increase in hepatocyte RDS (determined as the labelling index) after 7 days of treatment, as described above, the overall rate of cell proliferation would still be increased at longer time points due to the sustained increase in liver weight and hence the larger number of hepatocytes in the livers of PBO treated compared to control animals (Cohen, 2010; Elcombe et al., 2014; Lake, 2009, 2018; Yamada et at., 2021). Treatment with PBO for 79 weeks resulted in small increases in liver eosinophilic foci in male CD-1 mice given 300 mg/kg/day PBO and significant increases in the incidence of hepatocellular adenoma and in the combined incidence of hepatocellular adenoma and carcinoma in male mice given 100 and 300 mg/kg/day PBO. While liver eosinophilic foci were not observed in male mice given 100 mg/kg/day PBO for 79 weeks, no additional time points were studied. As described above, altered liver foci are considered to be the precursor lesion for subsequent liver tumor formation and hence would have been produced before the formation of liver tumors (Thoolen et al., 2012; Williams, 1997a,b).

Overall, there is a logical temporal response of the key and associative events for PBO-induced liver tumor formation in male CD-1 mice. Treatment with PBO results in effects on the identified key (hepatic CAR activation, increased hepatocyte RDS, and Cyp2b enzyme induction) and associative (liver hypertrophy and increased liver weights) events which occur before the onset of liver tumor formation. The time course for effects of PBO on the key and associative events is similar to that described for phenobarbital and a number of other nongenotoxic CAR activators which produce liver tumors in the mouse and/or rat (Cohen, 2010; Elcombe et al., 2014; Lake, 2018; La Rocca et al., 2017; LeBaron et al., 2014; Okuda et al., 2017a; Osimitz and Lake, 2009; Peffer et al., 2018; Tinwell et al., 2014; Wiemann, et al., 2019; Yamada, 2018; Yamada et al., 2009, 2021).

CARC Conclusions on Dose and Temporal Concordance: The dose-response and temporal association for key events leading to liver tumor formation are presented in **Table 40**.

Based on the evidence presented, the CARC concluded that there is strong concordance between key (and associative) events and the dose levels that produce tumors. The CARC also concluded that the key and associative events occur in a logical, time-dependent manner consistent with the proposed CAR MOA.

- **Key Event #1 (hepatic CAR activation and induction of Cyp2 enzymes).** *In vitro* data in male mice given PBO at dose levels of 30, 100 and 300 mg/kg/day for 14 days showed a dose related induction of microsomal Cyp2b protein levels, PROD enzyme activity, and Cyp2b10 mRNA expression levels at the dose levels of 30-100 mg/kg/day. Activation of CAR was also demonstrated in studies with C57BL/6J (wild type) and C57BL/6J CAR KO/PXR KO mice given a PBO dose level of 300 mg/kg/day for 14 days. Treatment with PBO markedly induced marked hepatic Cyp2b enzyme activity and Cyp2b10 mRNA levels in C57BL/6J wildtype mice, whereas

in CAR KO/PXR KO mice PBO treatment only produced a small increase in hepatic Cyp2b enzyme activity and a significant decrease in Cyp2b10 mRNA levels, most likely due to crosstalk between the two receptors. Treatment of 1000 mg/kg/day PBO for 4 weeks induced marked Cyp2b10 mRNA expression in male C3H/HeNCrlCrlj wildtype mice, whereas in C3H/HeNCrlCrlj CAR KO mice PBO treatment produced a smaller increase in Cyp2b10 but a larger increase in Cyp4a10 and Cyp3a11, indicating some crosstalk with PPARα and PXR receptors, respectively.

- **Key Event #2 (Increased cell proliferation).** The data indicate that at the tumorigenic doses, cell proliferation is increased in the initial 7 days after dosing before returning to levels similar to or slightly above the baseline proliferation levels, which is the expected proliferation response for CAR-mediated tumorigenesis. There was a marked, statistically significant increase in hepatocyte RDS in mice given 300 mg/kg/day PBO for 7 days. The treatment of CD-1 mice with 30 and 100 mg/kg/day PBO for 7 days resulted in elevated hepatocyte RDS compared to control, but were not statistically significant. These data were supported by studies with wildtype and CAR KO/PXR KO mice given 300 mg/kg/day and CAR KO mice given 1000 mg/kg/day, showing an increase in cell proliferation at 7 days only, with a more marked response in the wildtype mice than with the double KO or single KO mice.

- **Associative Events #1 and #2 (increased liver weight and liver hypertrophy).** Increased absolute/relative liver weights were observed after 7, 14 and 42 days in CD-1 male mice, as well as male C57BL/6J wildtype mice, and after 79 weeks of treatment in both male and female CD-1 mice treated with 300 mg/kg/day PBO. No effect on liver weight was seen in male PXR KO/CAR KO mice after 7 or 14 days given 300 mg/kg/day PBO. No changes in absolute liver weights were seen at 100 mg/kg/day for the shorter time points, but a statistical increase in relative liver weight was seen at 7 and 42 days. Dose related statistically significant increased absolute/relative liver weights were seen following 78 weeks exposure to PBO at 100 and 300 mg/kg/day in both male and female CD-1 mice.

  Hepatocyte hypertrophy was observed in male CD-1 mice given 300 mg/kg/day PBO for 7, 14, and 42 days and after 79 weeks of treatment; and in male and female mice given 100 mg/kg/day PBO for 42 days and after 79 weeks of treatment. Increased liver hypertrophy was seen at 7 and 14 days at 300 mg/kg/day PBO in male C57BL/6J wildtype mice, but there was no evidence of liver hypertrophy seen in the C57BL/6J PXR KO/CAR KO male mice at 300 mg/kg/day at these early timepoints.

- **Key Event #3 (clonal expansion leading to altered liver foci).** A small increase in eosinophilic foci was observed in male and female CD-1 mice treated with 300 mg/kg/day PBO for 79 weeks, but no evidence of foci in males was seen at 100 mg/kg/day, the tumorigenic dose in males. In addition, there was a slight increase in liver hyperplasia seen in both sexes at 300 mg/kg/day. Statistically increased altered eosinophilic foci were also seen at an earlier time point (27 weeks) in a study with male C3H/HeNCrlCrlj (wild-type) mice treated with a high dose of PBO (1000 mg/kg/day), with fewer foci seen in CAR KO mice, which may be due to crosstalk between CAR and PXR receptors.

- **Key Event #4 (liver tumor formation):** The CARC determined that liver tumors, driven by adenomas, were treatment related at ≥100 mg/kg/day in males and 300 mg/kg/day in females in a 78-week dietary study in mice.

The Agency has constructed **Table 40** in order to summarize the dose and temporal concordance of the key events in the proposed mode of action.

Table 40. Concordance Analysis of Key Events in the Proposed Mode of Action Associated with the Induction of Hepatocellular Adenomas and Carcinomas in Mice by PBO

Key Event	Dose Concordance	Temporal Concordance	Confidence/Uncertainty
1. Activation of Hepatic CAR	**MRID 51692503** **30, 100, 300 mg/kg/day (males only)** *Induction of Cyp2b expression, Cyp2b enzyme activity, Cyp2b10 mRNA levels PROD/EROD induction*	**MRID 51692503** **14 days** *Cyp mRNA induction* *PROD induction*	All mechanistic studies were conducted in males only. Registrant stated their rationale was that male mice were more susceptible to PBO tumors than female mice.
	MRID 51692505 **(CAR/PXR KO study)** **Only males tested** **300 mg/kg/day (Males only)** *PBO markedly induced Cyp2b enzyme activity and Cyp2b10 mRNA levels in C57BL/6J mice, but only produced a small increase in microsomal PROD activity and a significant decrease in hepatic Cyp2b10 mRNA levels in CAR KO/PXR KO mice.*	**MRID 51692505** **14 days** *Cyp mRNA induction* *PROD induction*	
	MRID 52376003 **Sakamoto et al. 2013** **1000 mg/kg/day (males)** *In wild-type mice, treatment with PBO increased hepatic mRNA expression levels of Cyp2b10 > Gadd45beta > Cyp3a11 > CYP reductase > Cyp1a2; In CARKO mice, treatment with PBO increased hepatic*	**MRID 52376003** **Sakamoto et al. 2013** **4 weeks** *Increased hepatic mRNA expression of Cyp2b10 greater in WT than KO mice*	

Table 40. Concordance Analysis of Key Events in the Proposed Mode of Action Associated with the Induction of Hepatocellular Adenomas and Carcinomas in Mice by PBO

Key Event	Dose Concordance	Temporal Concordance	Confidence/Uncertainty
	mRNA expression levels of Cyp4a10 > Cyp2b10 > Gadd45beta > Cyp1a1 > Cyp3a11 ≈ CYP reductase > Cyp1a2;		
2. Increased Cell Proliferation	**MRID 51692502 (males only)** **30 mg/kg/day** *-Increased BrdU LI (113%) not s.s.* **100 mg/kg/day** *-Increased relative liver weights* *-Increased BrdU LI (122%) not s.s.* **300 mg/kg/day** *-Increased BrdU LI (531%)* *-Increased absolute and relative liver weights* *-Increased hypertrophy*	**MRID 51692502** **7 days** *Increased BrdU LI* **7 days** *Increased relative liver weight* *Increased BrdU LI* **7 days** *-Increased BrdU LI* *-Increased abs/rel liver weights* *-Hepatocellular hypertrophy* **14 days** *Increased abs/rel liver weights* *Hepatocellular hypertrophy*	Associative events (increased liver weight and hypertrophy) are included
	MRID 51692504 **300 mg/kg/day (M/F)** *Wildtype mouse: Increased BrdU LI (6-fold)* *PXR/CAR KO mouse: Increased BrdU LI (4-fold)* Increased abs/rel liver weight	**MRID 51692504** **7 days** *-Increased BrdU LI wildtype and KO mice* *-Increased abs/rel liver wt in wildtype mice* *-Increased hepatocellular hypertrophy wildtype only* **14 days** *Increased abs/rel liver weight in wildtype mice* *Increased rel liver wt KO mice (<10%)*	
	MRID 52376008	**MRID 52376008**	

Table 40. Concordance Analysis of Key Events in the Proposed Mode of Action Associated with the Induction of Hepatocellular Adenomas and Carcinomas in Mice by PBO

Key Event	Dose Concordance	Temporal Concordance	Confidence/Uncertainty
	0, 10, 30, 100, 300 mg/kg/day for 7 and 42 days) males only **100 mg/kg/day** *-Increased relative liver weight* *-Increased liver hypertrophy* **300 mg/kg/day** *-Increased Labeling Index at 300 mg/kg/day only (348%)g* *Increased relative liver weight* *-Increased liver hypertrophy* **MRID 42903701** **100 mg/kg/day** *-Increased abs/rel liver weights in males and females* *-Increased liver hypertrophy in males* **300 mg/kg/day** *-Increased abs/rel liver weights in males and females* *-Increased hypertrophy in males and females* **MRID 52376003** *Male wildtype and CAR knockout (K)O mice* ***1000 mg/kg/day*** *-increased labelling index wildtype* *-increased abs/rel liver weights* *-increased hypertrophy (more severe in wildtype vs KO)*	**42 days** *Increased relative liver weight* *-increased liver hypertrophy* **7 days only** *-Increase Labeling Index* *-Increased relative liver weight* *-Increased liver hypertrophy* **42 days** *Increased relative liver weight* *-Increased liver hypertrophy* **MRID 42903701** **78 weeks** *-Increased abs/rel liver weights in males and females* *-increased hypertrophy* **MRID 52376003** *1 week (1000 mg/kg/day)* *Increased labelling index wildtype, but not at 4 weeks* *1, 4, and 27 weeks* *-Increased abs/rel liver weights* *4 and 27 weeks* *Increased hypertrophy in wildtype and KO (more*	

Table 40. Concordance Analysis of Key Events in the Proposed Mode of Action Associated with the Induction of Hepatocellular Adenomas and Carcinomas in Mice by PBO

Key Event	Dose Concordance	Temporal Concordance	Confidence/Uncertainty
		severe in wildtype than KO)	
3. Clonal Expansion Leading to Altered Hepatic Foci	**MRID 52376003** **1000 mg/kg/day** *-Eosinophilic foci in male mice*	**MRID 52376003** **27 weeks** *- Eosinophilic foci in males*	
	MRID 42903701 **300 mg/kg/day** *-Eosinophilic foci in males and females*	**MRID 42903701** **78 weeks** *-Eosinophilic foci in males and females*	
4. Liver Tumor Formation	**MRID 42903701** ≥100 mg/kg/day (M) 300 mg/kg/day (F)	**MRID 42903701** **78 Weeks**	

C. Strength, Consistency and Specificity

Extracted from the Registrant's MOA white paper (MRID 51692501):

> *The treatment of male CD-1 mice with PBO results in a pleiotropic response. The hepatic effects of PBO included increased liver weight with morphological evidence of hypertrophy, a stimulation of hepatocyte RDS and induction of Cyp2b subfamily enzymes. At the carcinogenic dose levels of 100 and 300 mg/kg/day PBO effects on these key and associative events were observed in the livers of male mice after short-term treatment, whereas liver tumors were only observed after chronic treatment. As described above, there is a logical temporal sequence where effects on the identified key and associative events are observed before PBO-induced mouse liver tumor formation.*
>
> *The relationship between the observed hepatic effects to PBO treatment in male CD-1 mice was demonstrated in recovery studies where the effects of PBO on liver weight and morphology, hepatocyte RDS, and induction of Cyp2b subfamily enzyme and mRNA levels were essentially fully reversible after the cessation of PBO treatment. Previous studies in mice and/or rats have demonstrated that the effects of phenobarbital and other known nongenotoxic CAR activators (e.g. fluopyram, metofluthrin, momfluorothrin, natural pyrethrins, nitrapyrin, sulfoxaflor) on liver hypertrophy,*

hepatocyte RDS, and induction of CYP enzymes are reversible on the cessation of treatment (Elcombe et al., 2014; Isenberg et al., 2001; Lake, 2018; Lake et al., 1978; La Rocca et al., 2017; LeBaron et al., 2014; Okuda et al., 2017a; Osimitz and Lake, 2009; Tinwell et al., 2014; Yamada et al., 2009, 2021). There are thus strong similarities between the proposed MOA for PBO-induced mouse liver tumor formation and the MOAs previously established for liver tumor formation by a number of other known nongenotoxic CAR activators.

CARC Conclusions on Strength, Consistency and Specificity: The data supporting the key events outlined for the proposed PBO CAR MOA are robust and the effects are replicated in several different mechanistic studies, supporting strength and consistency. When taken together, the mechanistic studies for CD-1 male mice clearly demonstrate a dose-related increase in the Cyp2b/CAR-associated mRNA expression level and associated increase in specific Cyp2b protein (Cyp2b10) and enzymatic activity (PROD). These results are consistent with the activation of the CAR nuclear receptor. In addition, analysis of hepatocellular proliferation indicates a clear, threshold, dose-related induction of S-phase DNA synthesis. Both of these key events were demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., C57BL/6J wildtype and C57BL/6J knockout, CAR KO/PXR KO), where a less robust response in CAR activity (gene or protein expression of Cyp2b10) or increase in hepatocellular proliferation was seen at the carcinogenic dose levels. Furthermore, the Cyp2b/CAR-associated gene expression and protein data from these MOA experiments in mice defines a very specific PBO CAR MOA while, simultaneously acknowledging some crosstalk, but ultimately ruling out other nuclear receptor-mediated MOAs as the primary MOA, such as PPAR-α or AhR agonism.

D. Biological Plausibility and Coherence

Extracted from the Registrant's MOA white paper (MRID 51692501):

The liver is the most common site of tumor formation in mouse and rat cancer bioassays (Gold et al., 2001; Huff et al., 1991). As described below, rodent liver tumors can be produced by a genotoxic MOA and by various nongenotoxic MOAs. The proposed MOA for PBO-induced mouse liver tumor formation is biologically plausible and is consistent with our current understanding of rodent liver tumor formation by nongenotoxic mitogenic agents that can activate nuclear receptors such as CAR and PPARα (Cohen, 2010; Corton et al., 2014, 2018; Elcombe et al., 2014; Lake, 2009, 2018; Yamada, 2018; Yamada et al., 2021). Mitogenic CAR and PPARα activators increase cell proliferation in rodent liver which can create an environment where spontaneously initiated cells have a greater chance to survive and divide, ultimately resulting in the formation of liver tumors.

PBO is predominantly a CAR activator in mouse liver and the proposed MOA for PBO-induced mouse liver tumor formation is similar to the MOAs established for phenobarbital and a number of other nongenotoxic CAR activators which produce liver tumors in the mouse and/or rat (Cohen, 2010; Elcombe et al., 2014; Lake, 2018; Peffer et al., 2018; Yamada, 2018; Yamada et al., 2021). Direct experimental evidence that PBO is predominantly a CAR activator in mouse liver was obtained in the studies with CAR

KO/PXR KO mice, where in contrast to effects in C57BL/6J (wild type) mice, treatment with PBO only produced a small increase in microsomal PROD activity and a significant decrease in hepatic Cyp2b10 mRNA levels in CAR KO/PXR KO mice. In addition, the treatment of male CD-1 mice with PBO produced a more marked effect on markers of CAR activation (i.e. Cyp2b subfamily enzyme activity and mRNA levels) than on the induction of other CYP subfamily enzymes.

CARC Conclusions on Biological Plausibility and Coherence: The postulated MOA for liver tumors in mice after exposure to PBO is considered biologically plausible and coherent. The key events outlined are largely consistent with the published scientific literature accounts of non-genotoxic mitogenic liver carcinogens. In addition, the specificity for the MOA was demonstrated for PBO using genetically engineered mouse models. PBO treated CAR KO/PXR KO mice did not demonstrate the CAR-mediated hepatic effects to the extent observed in treated wildtype mice. These data are consistent with the known MOA for PB and other CAR activators.

E. Alternative Modes of Action

Extracted from the Registrant's MOA white paper (MRID 51692501):

Many chemicals have been shown to increase the incidence of liver tumors in bioassays performed in the mouse and/or rat (Gold et al., 2001; Huff et al., 1991). Rodent liver tumors can be produced by a genotoxic MOA and by a number of nongenotoxic MOAs including both non-receptor mediated (e.g. cytotoxicity, metal overload) and receptor mediated (e.g. CAR activation, PPARα activation) MOAs (Cohen, 2010; Cohen and Arnold, 2011; Corton et al., 2014, 2018; Elcombe et al., 2014; Holsapple et al., 2006; Lake 2009, 2018; Meek et al., 2003; Yamada, 2018). The studies described in this document clearly demonstrate that PBO produces mouse liver tumors by a MOA involving CAR activation. As discussed below, other alternative MOAs for PBO-induced mouse liver tumor formation are not plausible and hence can be excluded.

Genotoxicity

Mutagenesis is always one possible MOA for rodent liver tumor formation. The genotoxicity of PBO has been reviewed (Butler et al., 1996; USEPA, 1995). PBO was shown not be mutagenic in a range of short-term tests (Butler et al., 1996; Kawachi et al., 1980; Rosenkrantz et al., 1990; White et al., 1977). These studies included a lack of effect of PBO on unscheduled DNA synthesis both in rat hepatocytes (Butler at al., 1996) and in human liver slices (Beamand et al., 1996).

The lack of genotoxicity of PBO has also been demonstrated in a study employing the gpt delta rat model to detect chemical carcinogens (Matsushita et al., 2013). PBO at a high dietary level of 12000 ppm for 4 weeks did not increase mutant frequencies of reporter genes in this model, thus confirming that PBO is not a genotoxic agent. Moreover, in the gpt delta rat model both PBO and phenobarbital increased glutathione S-transferase placental form positive foci after partial hepatectomy followed by a single dose of the genotoxic agent diethylnitrosamine. Based on the postulated MOA for PBO-induced mouse liver tumor formation, both PBO and phenobarbital would be expected to be

promoters in this rodent experimental initiation/promotion model. The results of this study thus support the proposed MOA for PBO-induced mouse liver tumor formation.

Overall, the data demonstrate that PBO is not a genotoxic agent and therefore produces mouse liver tumors by a nongenotoxic MOA.

Cytotoxicity

Liver tumors can be produced in rodents by cytotoxic agents such as chloroform where prolonged cytotoxicity results in a sustained regenerative hyperplasia, ultimately resulting in liver tumor formation (Cohen, 2010; Meek et al., 2003). Key events for this MOA include both sustained liver injury and a sustained regenerative hyperplasia. While the treatment of rats and mice with high toxic (>MTD) doses of PBO can produce marked hepatotoxicity and subsequently liver tumors (Fujitani et al., 1992, 1993a,b; Takahashi et al., 1994a,b, 1997), such effects were not observed in the PBO mouse bioassay in CD-1 mice conducted by the PBTFII. In this study the treatment of male and female CD-1 mice with up to 300 mg/kg/day PBO was not associated with increased hepatic necrosis (Butler et al., 1998). A cytotoxic MOA is therefore excluded for PBO-induced mouse liver tumor formation at doses up to the MTD.

Activation of hepatic aryl hydrocarbon receptor (AhR)

In some older investigations the treatment of mice with single intraperitoneal doses of PBO (ranging from 104 to 400 mg/kg) was shown after 24 hours to result in induction of both hepatic Cyp1a1 and Cyp1a2 mRNA levels (Adams et al., 1993a,b; Ryu et al., 1996, 1997). However, in more recent dietary studies the treatment of male C3H/He mice with 200, 1000 and 5000 ppm PBO (around 48, 207 and 882 mg/kg/day, respectively) for 7 days had no significant effect on hepatic Cyp1a1 mRNA levels (Sakamoto et al., 2015). In another study, the treatment of male C3H/He mice with 5000 ppm PBO for 4 weeks had no effect on hepatic Cyp1a1 mRNA levels, whereas some increase in Cyp1a2 mRNA levels was observed (Sakamoto et al., 2013). In the PBTFII studies with male CD-1 mice (the bioassay strain) treatment with 30-300 mg/kg/day PBO and 500 ppm sodium phenobarbital produced significant increases in hepatic Cyp1a2 mRNA levels and in microsomal EROD activity and Cyp1a protein content. The increases in Cyp1a2 mRNA levels and in microsomal EROD activity and Cyp1a protein content do not reflect AhR activation. The relatively small increase in microsomal EROD activity is most unlikely to represent induction of Cyp1a enzymes, but rather that this CYP enzyme substrate is also metabolized by other CYP enzymes (including Cyp2b and Cyp2c subfamily enzymes) which are induced by CAR activators such as phenobarbital (Burke et al, 1994). As noted above, in terms of hepatic Cyp1a1 induction, while some increases have been reported in the older literature in mice in single acute dose studies, no induction of hepatic Cyp1a1 mRNA levels has been observed in more recent dietary studies where PBO was administered to mice at dose levels of 200-5000 ppm for 7 days or at 5000 ppm for 4 weeks (Sakamoto et al., 2013, 2015). Indeed, the known CAR activator sodium phenobarbital was shown to produce a small increase in mouse hepatic Cyp1a1 mRNA levels after 4 weeks treatment at a dietary level of 500 ppm (Sakamoto et al., 2013). However, this effect is not attributable to AhR activation as while Cyp1a1 mRNA is a

sensitive marker of AhR activation, it lacks specificity as many compounds which increase Cyp1a1 mRNA levels in vivo have been shown not to bind to or activate AhR in vitro (Hu et al., 2007). With respect to Cyp1a2 induction, treatment with both PBO and sodium phenobarbital increased Cyp1a2 mRNA levels in the PBTFII MOA studies (Table 8). Hepatic Cyp1a2 mRNA levels were also increased in male C3H/He mice given 5000 ppm PBO and 500 ppm sodium phenobarbital for 4 weeks (Sakamoto et al., 2013). Several studies have demonstrated that the known CAR activator phenobarbital can increase mouse hepatic Cyp1a2 mRNA levels by a non AhR-dependent mechanism (Corcos et al., 1998; Sakuma et al., 1999; Zaher et al., 1998). In the PBTFII study, the increase in microsomal Cyp1a protein content is thus attributable to the induction of Cyp1a2 protein, as Cyp1a1 protein is not normally expressed in mouse liver. Overall, the results of the PBTFII studies and the available literature demonstrate that PBO does not activate AhR in mouse liver.

<u>Activation of hepatic PXR</u>
Treatment with PBO produced some increases in hepatic Cyp3a11 mRNA levels and in microsomal T6βOH activity and Cyp3a protein levels, but the effects were less marked than those observed on the Cyp2b markers (Tables 7 and 8) and may be due to crosstalk between the hepatic CAR and PXR receptors (Omiecinski et al., 2011; Yoshinari et al., 2008). For example, studies with CAR KO and PXR KO mice (i.e. mice lacking hepatic PXR) have suggested that the induction of Cyp3a11 by phenobarbital is mainly due to activation of CAR (Scheer et al., 2008). Overall, it is considered that the hepatic effects of PBO in mouse liver are predominantly due to CAR activation.

<u>Activation of hepatic PPARα</u>
In the PBTFII study with male CD-1 mice (the bioassay strain), treatment with PBO produced a biphasic effect on markers of PPARα activation. Treatment with 100 mg/kg/day PBO significantly increased hepatic Cyp4a10 mRNA levels together with microsomal Cyp4a-dependent LA12OH activity and Cyp4a protein content; whereas treatment with 300 mg/kg/day PBO had no effect on microsomal LA12OH activity and produced significant decreases in hepatic Cyp4a10 mRNA levels and microsomal Cyp4a protein content. The biphasic effect on markers of hepatic PPARα activation following treatment with PBO is due to known interactions between hepatic CAR and PPARα receptors which have been previously demonstrated to modulate each other's activity (Maglich et al. 2009; Tamasi et al., 2009; Ueda et al. 2002; Yoshinari et al., 2008). For example, the treatment of mice with the potent mouse CAR activator 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene resulted in a down regulation of hepatic PPARα and Cyp4a14 mRNA levels, whereas the basal level of Cyp4a14 mRNA was greater in mice lacking CAR than in normal mice (Maglich et al. 2009). In other studies PB was shown to induce Cyp4a10 and Cyp4a14 mRNA levels in either CAR KO mice or CAR KO/PXR KO mice, but not in normal mice (Tamasi et al., 2009; Ueda et al. 2002). As such, the present data suggest that in CD-1 mice the marked induction of CAR following treatment with PBO leads to a suppression of PBO activation of PPARα. This conclusion is supported by other studies where treatment of male mice with 5000 ppm PBO for 7 days or 4 weeks was shown to markedly induce hepatic Cyp4a10 mRNA levels in CAR KO mice, but to

have little or no effect in C3H/He mice (Sakamoto et al., 2013, 2015). In the present study the treatment of both male C57BL/6J and CAR KO/PXR KO mice with PBO for 7 days resulted in a significant increase in hepatocyte RDS (Table 9). However, the effect of PBO on hepatocyte RDS in CAR KO/PXR KO mice does not detract from the postulated MOA. As demonstrated by several studies (Maglich et al. 2009; Tamasi et al., 2009; Ueda et al. 2002; Yoshinari et al., 2008), in the absence of hepatic CAR PBO can activate PPARα, with PPARα activators being known mitogenic agents in rodent liver (Corton et al., 2014, 2018; Lake, 2009; Yamada et al., 2021). Indeed the treatment of CAR KO/PXR KO mice, but not C57BL/6J (wild type) mice, with PBO resulted in significant increases in some markers of PPARα activation, namely hepatic PCoA activity and acyl-CoA oxidase and Cyp4a10 mRNA levels. The stimulation of hepatocyte RDS by PBO in CAR KO/PXR KO mice is thus attributable to PPARα activation in the absence of suppression of this nuclear receptor by CAR. Overall, in normal mice (i.e. CD-1 mice) the hepatic effects of PBO are predominantly mediated through CAR and not PPARα activation.

Other MOAs

Other MOAs for rodent liver tumor formation include hormonal perturbation, infection, immunosuppression, and metal overload (Cohen, 2010; Cohen and Arnold, 2011). There is no evidence that these MOAs are associated with PBO-induced mouse liver tumor formation.

Summary of evaluation of alternative MOAs

A number of in vitro and in vivo tests have demonstrated that PBO is not a genotoxic agent and hence PBO-induced mouse liver tumor formation is thus due to a nongenotoxic MOA. At up to MTD doses, PBO does not produce liver damage in the mouse, and hence the MOA for PBO- induced mouse liver tumor formation is not due to cytotoxicity. While PBO can induce CYP enzymes in various subfamilies in mouse liver, PBO has the greatest effect on markers of Cyp2b subfamily enzymes, and hence PBO is predominantly a CAR activator in mouse liver. This was confirmed by the studies with C57BL/6J and CAR KO/PXR KO mice, where in the CAR KO/PXR KO mice the effects of PBO on hepatic Cyp2b10 enzyme activity and mRNA levels were either much reduced or abolished. Overall, in normal (wild type) mice, while treatment with PBO can activate PPARα, the much greater activation of CAR leads to a suppression of effects on PPARα markers.

CARC Conclusions on Alternative MOAs: Alternative MOAs (*i.e.,* genotoxicity, cytotoxicity, hormonal perturbation, immunosuppression, infection, metal overload, increased apoptosis, mitogenesis induced by other nuclear receptors such as hepatic aryl hydrocarbon receptor AhR, hepatic PXR, and hepatic PPARa) have been adequately ruled out as the primary MOA leading to tumor formation following PBO exposure. PBO did not cause genetic damage when tested in a series of *in vitro* assays such that the overall weight of evidence suggests that PBO does not have genotoxic potential. Treatment of male and female CD-1 mice up to 300 mg/kg/day PBO for 78 weeks was not associated with increased hepatic necrosis, therefore, a cytotoxic MOA can therefore be excluded for PBO-induced mouse liver tumor formation. While PBO can induce CYP enzymes in various subfamilies in mouse liver, PBO has the greatest effect on markers of Cyp2b subfamily enzymes, therefore, PBO is

predominantly a CAR activator in mouse liver. The MOA studies in mice demonstrated a specific, dose-related increase in the *Cyp2b*/CAR mRNA induction with associated increases in Cyp2b protein and enzymatic activity (PROD). Furthermore, CARKO/PXRKO animals further supported specificity for activation of the CAR/PXR receptor. This was confirmed by the studies with C57BL/6J and CAR KO/PXR KO mice, where in the CAR KO/PXRKO mice the effects of PBO on hepatic Cyp2b10 enzyme activity and mRNA levels were either much reduced or abolished. The mechanistic studies showed that the treatment of male CD-1 mice with PBO produced a more marked effect on markers of CAR activation (i.e. Cyp2b subfamily enzyme activity and mRNA levels) than on the induction of other CYP subfamily enzymes, such as PXR. The smaller increases seen in hepatic Cyp3a11 mRNA levels and PROD activity (markers for PXR activation) in male CD-1 mice may be due to crosstalk between the hepatic CAR and PXR receptors. Additionally, the mechanistic studies also showed PBO did not produce any marked effects on the markers of peroxisome proliferation. C57Bl/6J wildtype and CAR KO/PXR KO mice fed 300 mg/kg/day PBO in the diet for 14 days showed no increase in Cyp4a10 mRNA levels in wildtype mice (showing a suppression of effects on PPARα) but showed a significant increase in Cyp4a10 mRNA levels in the PXR KO/CAR KO mice. The increased Cyp1a2 markers, indicative of AhR activation, seen in CD-1 mice as well as the wildtype and CAR/PXR KO mice is likely due to crosstalk. A study by Ryu *et al*. 1996 showed that AhR knockout mice treated with 200 mg/kg PBO demonstrated increased Cyp1a2 induction (increased Cyp1a2 mRNA levels in AhR KO mouse liver), without the induction of Cyp1a1, which suggests the possibility of an AhR-independent mechanism of Cyp1a2 induction, likely due to crosstalk. Overall, CARC considered that the hepatic effects of PBO in the mouse liver are predominantly due to CAR activation.

F. Uncertainties, Inconsistencies and Data Gaps

Extracted from the Registrant's MOA white paper (MRID 51692501):

> *While PBO produced liver tumors in both male and female CD-1 mice, the MOA studies conducted by the PBTFII focused on just male CD-1 mice. However, as shown in Table 5a male CD-1 mice are more susceptible to PBO-induced liver tumor formation with, in contrast to female mice, a significant increase in the incidence of hepatocellular adenoma being observed in male mice given 100 mg/kg/day. In addition, the incidence of hepatocellular adenoma observed at a PBO dose level of 300 mg/kg/day was also much lower in female than in male CD-1 mice. While PBO can produce liver tumors in mice at up to MTD levels, PBO does not produce liver tumors in the Sprague-Dawley rat at up to MTD levels; hence no MOA studies were performed in rats. The lack of MOA studies in female CD-1 mice and in male and female Sprague-Dawley rats is thus not considered to constitute a data gap. In two 7 days studies, PBO at a dose level of 100 mg/kg/day was shown to increase hepatocyte RDS in male CD-1 mice to around two-fold of control levels, but due to inter-animal variation these increases were not statistically significant. However, these increases are considered to be biologically significant and hence do not represent a data gap, as treatment with 100 mg/kg/day PBO did not result in a marked increase in liver tumor incidence in male mice and produced no significant increase in liver tumor incidence in female mice. In the CD-1 mouse bioassay the treatment of male and female CD-1 mice with 300 mg/kg/day PBO for 79 weeks produced only small increases in eosinophilic foci. Altered liver foci are only observed in*

chronic studies at some time points, with altered liver foci considered to be the precursor lesion for subsequent liver tumor formation (Thoolen et al., 2012; Williams, 1979a,b). The lack of an effect of PBO on the incidence of eosinophilic foci in male mice given 100 and 300 mg/kg/day PBO and in female mice given 300 mg/kg/day PBO is thus not considered to constitute a data gap and does hence not detract from the proposed MOA.

CARC Conclusions on Uncertainties, Inconsistencies and Data Gaps: PBO produced treatment related liver tumors at ≥100 mg/kg/day in male mice and at 300 mg/kg/day in female mice. The mechanistic studies were conducted with male mice only since the tumorigenic dose in males is lower than in females, showing males to more susceptible to PBO-induced liver tumor formation than female mice. There are no mode of action studies available in female mice. An article by Peffer *et al.* 2018 states than in cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient. In support of this, the CARC also notes the lack of sex differences in the PBO database. The CARC concluded that the lack of mode of action data in female mice does not detract from the overall plausibility of that this mode of action is also operative in female mice.

The CARC also noted an inconsistency in the proposed MOA with Key Event #3 (clonal event leading to altered liver foci), as liver foci was observed at only 300 mg/kg/day in the 78-week mouse study and not at 100 mg/kg/day, a tumorigenic dose in males. In addition, there was a slight increase in liver hyperplasia seen in both sexes at 300 mg/kg/day. While Key Event #3 remains an inconsistency, the CARC noted that increases in foci are not always able to be observed. Peffer *et al*. 2018 noted that an increase in eosinophilic foci is most commonly observed after long-term administration of CAR activators but a documented increase in the number of altered foci in rodent livers may not be identified in a particular study because of the timing of sacrifice(s) and the intrinsic characteristics of the chemical of interest.

G. Relevance of MOA to Humans

Are the key events in the animal mode of action for liver tumors plausible in humans?

The Registrant has proposed in their white paper (MRID 51692501) to evaluate the human relevance of the proposed MOA for liver tumors using the IPCS Human Relevance Framework (Boobis, *et al*., 2006). In this framework, a series of three fundamental questions is posed in evaluating the human relevance of an animal MOA for tumor formation.

Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?

In response to this question, the Registrant suggests that a robust MOA has been established for PBO-induced formation of liver tumors in male and female mice due to CAR activation, citing the mechanistic studies submitted in support of the proposed MOA. Further, the Registrant suggests that alternative MOAs can be excluded from consideration.

Excerpt from Registrant-submitted white paper (MRID 51692501) :

The answer to this question is clearly yes. As described above, there is clear evidence to

support a robust MOA for PBO-induced mouse liver tumor formation due to CAR activation. The postulated MOA is consistent with MOAs previously established for phenobarbital and several other nongenotoxic CAR activators that produce liver tumors in rodents (Cohen, 2010; Elcombe et al., 2014; Holsapple et al., 2006; Lake, 2018; La Rocca et al., 2017; LeBaron et al., 2014; Okuda et al., 2017a; Osimitz and Lake, 2009; Peffer et al., 2018; Tinwell et al., 2014; Wiemann et al., 2019; Yamada, 2018; Yamada et al., 2009, 2021). As described above, based on the available data, alternative MOAs for PBO-induced mouse liver tumor formation can be excluded.

Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?
MRID 51692506. Elcombe, B. and Vardy, A. 2017. Piperonyl Butoxide: MOA Phase III – Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male CD-1 Mouse Hepatocytes

MRID 51692507. Elcombe, B and Vardy, A. 2017.Piperonyl Butoxide: MOA Phase III – Cytochrome P450 Enzyme and Replicative DNA-Synthesis Induction in Cultured Male and Female Human Hepatocytes

In response to this question, the Registrant conducted a series of studies that investigated the effect of PBO and sodium phenobarbital RDS in cultured male CD-1 mouse (MRID 51692506) and in male and female human primary hepatocytes (MRID 51692507). Mouse and human hepatocytes were also treated with epidermal growth factor (EGF) as a positive control for the RDS studies. The cytotoxicity of PBO and sodium phenobarbital to mouse and human hepatocytes was determined by measuring hepatocyte ATP content and the effects of treatment with PBO and sodium phenobarbital on some CYP mRNA levels was also investigated.

The results of the treatment of both male mouse and male and female human hepatocytes with 5-500 µM PBO for 4 days are presented in **Table 41** and **Table 42**, respectively.

As shown in **Table 41**, PBO at 10, 20, and 50 µM moderately reduced ATP levels to 71%, 61% and 60% of control values, respectively. However, severe cellular toxicity was measured at ≥200 µM, where ATP levels were <2% of control. Treatment with NaPB had no biologically significant effect on ATP levels (9-13% of controls) at concentrations up to 1000 µM.

Treatment with ^{3}5 µM PBO caused strong concentration-dependent, statistically-significant increases in replicative DNA synthesis as determined by the S-phase labelling index, up to 5.9- fold at 20 µM (**Table 41**). A statistically significant increase in the S-phase labelling index was also noted at 50 µM PBO (5.0-fold). However, there was a significant reduction in the S-phase labelling index following administration of PBO at 200 µM, coupled with cytotoxicity as determined by visual assessment and ATP depletion data. The number of hepatocytes remaining on the culture plate were reduced at this concentration of PBO, although those remaining were morphologically normal. However, the cytotoxicity measured at 500 µM was too severe for the S-phase labelling index to be determined. Treatment with NaPB or EGF resulted in statistically significant increases in replicative DNA synthesis, as expected.

Treatment with 5 µM PBO resulted in a statistically significant increase in Cyp3a11 mRNA levels (1.4-

Xcontrol); whereas the increase in Cyp2b10 mRNA levels to 1.6-X control was not statistically significant, owing to variation between the replicates. The treatment of male CD-1 mouse hepatocytes with ≥20 µM PBO resulted in a marked statistically significant reduction in Cyp2b10 and Cyp3a11 mRNA levels. Treatment with NaPB produced concentration-dependent increases in Cyp2b10 and Cyp3a11 mRNA levels. Cyp2b10 mRNA levels were significantly increased to 1.7- and 2.3-fold by treatment with 100 and 1000 µM NaPB, respectively, whereas Cyp3a11 mRNA levels were significantly increased to 1.6-fold control by treatment with 1000 µM NaPB.

Table 41. Effect of treatment of male CD-1 mouse hepatocytes with PBO and NaPB on cytotoxicity, RDS, and Cyp2b10 and Cyp3a11 mRNA levels

Treatment[a]	Cytotoxicity (ATP content (luminescence units)[b] Mean±SD (% of control)	Hepatocyte RDS[b] Mean±SD (% of control or fold change from control)	Cyp mRNA levels[c]	
			Cyp2b10 mRNA Mean±SD (% of control or fold change from control)	Cyp3a11 mRNA Mean±SD (% of control or fold change from control)
Vehicle Control (0.1% v/v DMSO)	636307±15523 (100)	1.34 ± 0.33 (100)	1.00 ± 0.19 (100)	1.00 ± 0.09
PBO 5 µM	550593 ± 16143*** (87)	3.44 ± 0.28*** (257)	1.58 ± 0.35 (1.58)	1.40 ± 0.10** (1.4-fold)
PBO 10 µM	451026 ± 9897*** (71)	4.61 ± 0.48*** (344)	0.79 ± 0.06 (20)	1.31 ± 0.29 (1.31-fold)
PBO 20 µM	388820 ± 18112*** (61)	7.91 ± 0.65*** (590)	0.04 ± 0.03*** (4)	0.23 ± 0.09*** (23)
PBO 50 µM	381907 ± 12522*** (60)	6.72 ± 0.48*** (501)	0.01 ± 0.01*** (1)	0.10 ± 0.02*** (10)
PBO 200 µM	10185 ± 3065*** (1.6)	0.64 ± 0.28** (48)	0.08 ± 0.06** (8)	0.17 ± 0.08*** (17)
PBO 500 µM	947 ± 124*** (0.15)	n.d.[d]	0.11 ± 0.17** (11)	0.02 ± 0.04*** (2)
NaPB 10 µM	577847 ± 26202*** (91)	2.01 ± 0.11** (1.5-fold)	1.03 ± 0.03	0.98 ± 0.06
NaPB 100 µM	553202 ± 13573*** (87)	2.63 ± 0.37*** (1.96 fold)	1.72 ± 0.08** (1.72-fold)	1.14± 0.21
NaPB 1000 µM	551556 ± 6499*** (87)	1.88 ± 0.29* (1.40-fold)	2.33 ± 0.17*** (2.33 -fold)	1.63 ± 0.26* (1.63-fold)
EGF 25 ng/ml[e]	-	8.43 ± 1.47*** (629)	-	-

[a] Male CD-1 mouse hepatocytes were treated with 0 (control), 5-500 µM PBO, 10-1000 µM NaPB and 25 ng/ml EGF for 96 hours. To determine hepatocyte RDS BrdU was added to the culture medium for the last 72 hours of treatment. Values significantly different from control are: *p<0.5; ** p<0.01; ***p<0.001.

[b] Results are expressed as a percentage of the maximum amount of ATP released (i.e., the value of control cells) and S-phase labelling index mean percent of control means of five and six replicates for hepatocyte RDS and cytotoxicity, respectively.

[c] Results are fold control levels (means of three replicates). Data from Elcombe and Vardy (2017a)

[d] n.d. = not determined, due to high cytotoxicity at this concentration.

[e] The effect of 25 ng/ml EGF on cytotoxicity and Cyp mRNA levels in cultured male CD-1 mouse hepatocytes was not determined.

In marked contrast to male mice, treatment with ≥50 µM PBO caused cytotoxicity (as determined by depletion in ATP levels) in both male and female hepatocyte cultures. NaPB had no significant effect on ATP levels. The treatment of male and female human hepatocytes with PBO and sodium phenobarbital did not result in any significant increases in hepatocyte RDS (**Table 42**). Levels of RDS human hepatocytes were either significantly decreased or were undetectable after treatment with markedly cytotoxic concentrations of PBO (i.e. 200 and 500 µM).

There was some evidence of PBO-mediated induction of CYP2B6 and CYP3A4 mRNA levels in both the male and female human hepatocytes. Although there was no strong evidence of a concentration-dependency, CYP2B6 and CYP3A4 mRNA levels peaked at increases of 3.8-fold and 2.6-fold, respectively in male human hepatocytes. Some statistically significant increases in CYP2B6 and CYP3A4 mRNA levels were also observed in female human hepatocytes. These increases in CYP mRNA levels suggest that PBO may activate both the human CAR and the PXR. Treatment with NaPB resulted in statistically-significant increases in both CYP2B6 and CYP3A4 mRNAs in both sets of human hepatocytes, as expected.

The functional viability of the male mouse and male and female human hepatocytes used in these studies to a hepatocyte mitogen was confirmed by the effects of treatment with 25 ng/ml EGF where hepatocyte RDS values were significantly increased 629%, 1469% and 3509% compared to control, respectively (**Tables 41** and **42**). EGF produced a robust increase in replicative DNT synthesis, demonstrating that the test system could respond to a proliferative stimulus.

Table 42. Effect of treatment of male and female human hepatocytes with PBO and NaPB on cytotoxicity, RDS, and CYP2B6 and CYP3A4 mRNA levels					
Sex	Treatment[a]	Cytotoxicity (ATP assay)[b]	Hepatocyte RDS[b]	CYP mRNA levels[c]	
				CYP2B6	CYP3A4
Male	PBO 5 µM	104	92	3.52**	2.20**
	PBO 10 µM	94	100	3.29*	1.74
	PBO 20 µM	98	106	3.83*	2.58*
	PBO 50 µM	62***	94	2.92***	2.29**
	PBO 200 µM	5.2***	43**	3.55*	2.30
	PBO 500 µM	0.9***	n.d.[d]	n.d.	n.d.
	NaPB 10 µM	107	102	2.03	1.22
	NaPB 100 µM	103	90	2.90*	1.71*
	NaPB 1000 µM	92	82	9.92**	6.98**
	EGF 25 ng/ml[e]	-	1469***	-	-
Female	PBO 5 µM	95	117	2.02	2.05
	PBO 10 µM	96	104	1.36	1.39
	PBO 20 µM	94	104	1.36	1.65
	PBO 50 µM	76**	96	1.18	1.92*
	PBO 200 µM	11.4***	n.d.	2.95	3.63**
	PBO 500 µM	1.1***	n.d.	1.76*	1.94*
	NaPB 10 µM	115*	102	0.81	0.95
	NaPB 100 µM	112	98	1.16	1.20
	NaPB 1000 µM	95	91	2.80**	3.62**
	EGF 25 ng/ml[e]	-	3509***	-	-

[a] Male human (Caucasian donor aged 51 years) and female (Caucasian donor aged 52 years) hepatocytes were treated with 0 (control), 5-500 µM PBO, 10-1000 µM NaPB and 25 ng/ml EGF for 96 hours. To determine hepatocyte RDS BrdU was added to the culture medium for the last 72 hours of treatment. Values significantly different from control are: *p<0.05; ** p<0.01; ***p<0.001.

[b] Results are percentage of control levels (means of five and six replicates for hepatocyte RDS and cytotoxicity, respectively). Data from Elcombe and Vardy (2017b).

[c] Results are fold control levels (means of three replicates). Data from Elcombe and Vardy (2017b)

[d] n.d. = not determined, due to high cytotoxicity at this concentration.

[e] The effect of 25 ng/ml EGF on cytotoxicity and CYP mRNA levels in cultured male and female human hepatocytes was not determined.

Excerpt from Registrant-submitted white paper (MRID 51692501):

The answer to this question is clearly yes. A comparison of the effects of PBO in mice and humans on the key (i.e. activation of CAR, increased RDS, altered hepatic foci and liver tumors) and associative (i.e. liver hypertrophy and induction of Cyp2b enzymes) events for the proposed MOA for mouse liver tumor formation is shown in Table 43. As with mice, high doses of PBO would be expected to activate CAR in human liver. Indeed, as a marker for CAR activation, the treatment of human hepatocytes with PBO, like sodium phenobarbital, did result in some increases in CYP2B6 and CYP3A4 mRNA levels. Studies with phenobarbital and a number of other compounds have demonstrated that both CYP2B and CYP3A subfamily enzymes are

induced in human liver by interaction with both the CAR and PXR receptors (Hakkola et al., 2020; Maglich et al., 2002; Martignoni et al., 2006; Moore et al., 2003; Omiecinski et al., 2011; Pelkonen et al., 2008). In one in vivo study, the administration of a single oral 0.71 mg/kg PBO dose to eight male human subjects had no effect on antipyrine half-life determined two hours after the dose of PBO (Conney et al., 1972). However, additional studies with prolonged PBO treatment would be required to demonstrate if high doses of PBO could induce hepatic xenobiotic metabolism in humans.

In terms of human relevance, the key species difference is that while CAR activators are mitogenic agents in rodent liver, they do not stimulate RDS in human hepatocytes (Cohen, 2010; Elcombe et al., 2014; Lake, 2009, 2018, Yamada, 2018; Yamada et al., 2021). This was demonstrated in cultured male CD-1 mouse and male and female human hepatocytes in the present studies with PBO. The lack of effect of PBO on RDS in cultured human hepatocytes is in agreement with a number of studies with phenobarbital and other nongenotoxic mitogenic rodent CAR activators conducted in several different laboratories (Haines et al., 2018; Hirose et al., 2009; Kondo et al., 2020; Okuda et al., 2017b; Parzefall et al., 1991; Soldatow et al., 2016; Wiemann et al., 2019; Yamada et al., 2015, 2021). Additional data that rodent liver CAR activators do not stimulate RDS in human hepatocytes comes from in vivo investigations with chimeric mice with humanized livers. Studies with sodium phenobarbital and two other CAR activators have demonstrated that while such compounds can induce CYP enzymes in human hepatocytes of chimeric mice, they do not stimulate RDS (Okuda et al., 2017b; Yamada et al., 2014). Based on the lack of effect of PBO on RDS in human hepatocytes it is considered that the MOA for PBO-induced mouse liver tumor formation is qualitatively not plausible for humans. This conclusion is supported by epidemiological studies with phenobarbital, oxazepam and carbamazepine that demonstrate no increased risk of liver tumors after treatment for extended periods (Friedman et al., 2009; IARC, 2001; Iqbal et al., 2015; La Vecchia and Negri 2014; Stritzelberger et al., 2021). Finally, the chronic dietary exposure of the general human population to PBO is orders of magnitude lower than the PBO dose levels required to produce liver tumors in mice. Overall, the postulated MOA for PBO-induced liver tumor formation in mice is considered to be not plausible for humans on the basis of qualitative (also quantitative) differences between humans and mice.

Table 43. Comparison of key and associative events for PBO-induced mouse liver tumor formation in mice and humans (excerpted from Registrant submission MRID 51692501)

Key (KE) or associative (AE) event	Evidence in mice	Evidence in humans
Activation of CAR (KE)	Inferred from induction of Cyp2b enzymes *in vivo*. Demonstrated in *in vivo* studies with CAR KO/PXR KO mice	Probable at high doses[a]. Induction of CYP2B6 and CYP3A4 mRNA levels observed in cultured human hepatocytes
Induction of CYP2B subfamily enzymes (AE)	Direct experimental evidence *in vivo*	Probable at high doses[a]. Induction of CYP2B6 and CYP3A4 mRNA levels observed in cultured human hepatocytes
Hepatocyte hypertrophy (AE)	Direct experimental evidence *in vivo* (effects on relative liver weight and hepatocyte hypertrophy)	Possible at high doses[b]
Increased hepatocyte RDS (KE)	Direct experimental evidence *in vivo* and *in vitro* in cultured hepatocytes	Not predicted to occur[c]. Not observed in cultured male and female human hepatocytes.
Altered liver foci (KE)	Small increase in eosinophilic foci observed *in vivo*	Not predicted to occur[d]
Liver tumors (KE)	Increased incidence of hepatocellular adenomas observed *in vivo*	Not predicted to occur[e]

[a] Inferred from studies with phenobarbital and other hepatic CAR activators where induction of hepatic CYP enzymes has been demonstrated in *in vitro* studies with cultured human hepatocytes and also in *in vivo* studies in human subjects (Hakkola et al., 2020; Parkinson et al., 2004; Martignoni et al., 2006; Pelkonen et al., 2008).

[b] Inferred from studies with phenobarbital and other antiepileptic drugs where prolonged administration has been shown to produce evidence of liver hypertrophy in human subjects (Aiges et al., 1980; Pirttiaho et al., 1978, 1982).

[c] Many studies with phenobarbital and other nongenotoxic CAR activators have demonstrated that cultured human hepatocytes are refractory to the mitogenic effects of rodent CAR activators (Haines et al., 2018; Hirose et al., 2009; Kondo et al., 2020; Okuda et al., 2017; Parzefall et al., 1991; Soldatow et al., 2016; Wiemann et al., 2019; Yamada et al., 2015, 2021). In addition, in *in vivo* studies sodium phenobarbital and two other rodent CAR activators did not stimulate RDS in human hepatocytes of chimeric mice with humanized livers (Okuda et al., 2017b; Yamada et al., 2014).

[d] Rodent CAR activators (e.g. phenobarbital and other antiepileptic drugs) have not been reported to produce altered liver foci in human liver (Elcombe et al., 2014).

[e] Epidemiological studies with phenobarbital, oxazepam and carbamazepine have demonstrated that such compounds do not increase the incidence of liver tumors in human subjects (Friedman et al., 2009; IARC, 2001; Iqbal et al. 2015; La Vecchia and Negri, 2014; Stritzelberger et al., 2021).

Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

Excerpt from Registrant-submitted white paper (MRID 51692501):

"As described above, the MOA for PBO-induced mouse liver tumor formation is qualitatively not plausible for humans; hence it is not necessary to consider quantitative differences in either kinetic or dynamic factors between mice and humans. However, it is worthwhile considering human exposure to PBO. Chronic dietary exposure in the general US population has been reported to be approximately 0.027 mg/kg/day (US EPA, 2017).

Human exposure to PBO is thus over 3700 times lower than the lowest 100 mg/kg/day dietary level of PBO which produced liver tumors in male CD-1 mice. Hence, not only can the mouse MOA be considered not relevant to humans based on qualitative species differences, but the quantitative differences in exposure also render the MOA not relevant to humans."

CARC Conclusion on Human Relevance: Overall, the CARC determined that these data do not sufficiently support the lack of relevance of this MOA to humans. The human CAR receptor is activated by PBO as shown by the induction of CYP2B6 and CYP3A4 mRNA levels observed in cultured human hepatocytes from two human subjects in *in vitro* studies and, therefore, can be relevant to humans qualitatively. This *in vitro* study generally showed concordance between the mouse and human hepatocyte response to PBO in culture (cytotoxic and cyp induction of PBO) but did not show concordance with hepatocyte RDS (positive with mouse hepatocytes and negative with human hepatocytes). These data, with only two subjects, are not robust enough to show definitive differences between mice and humans.

Consistent with current HED policy and practice, the CARC considers the CAR MOA for PBO induced mouse liver tumor formation to be qualitatively and quantitatively plausible in humans.

H. CARC Conclusions on the Proposed MOA for Liver Tumors in CD-1 Mice

Overall, the CARC concluded that the Registrant's proposed MOA involving CAR-mediated development of hepatocellular tumors in male and female CD-1 mice was adequately supported under the IPCS MOA framework and the 2005 Guidelines for Carcinogenic Risk Assessment. Key Events #1 and #2 and Associative Events #1 and #2 were adequately supported and were demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., C57BL/6J wildtype and C57BL/6J knockout, CAR KO/PXR KO), where little or no CAR activity (gene or protein expression of Cyp2b10) or where reduced CAR activity (labeling index) was seen at 300 mg/kg/day in the double KO mice and where increased liver weight and hypertrophy were seen at 300 mg/kg/day in the wildtype mice but not in the double KO mice. Furthermore, the Cyp2b/CAR-associated gene expression and protein data as well as the increased cell proliferation data from these MOA experiments in mice define a very specific PBO CAR MOA as the dominant activator, while simultaneously acknowledging some crosstalk with other liver nuclear receptors. However, a primary role of other nuclear receptor-mediated MOAs for rodent hepatic carcinogens such as pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR-α), or aromatic hydrocarbon-responsive receptor (AhR) agonism was ruled out. Key Event #3 was partially supported (liver foci were seen at 300 mg/kg/day, but not at 100 mg/kg/day). The CARC concluded there is strong concordance between key (and associative) events and the dose levels that produce tumors. The CARC also concluded that the key and associative events occur in a logical, time-dependent manner consistent with the proposed CAR MOA. The MOA data was conducted in male mice only, however, the CARC did not consider this to be a significant data gap since males were more susceptible to PBO-induced liver tumor formation than female mice. The CARC considered the data generated in one sex representative of the response in both males and females and does not detract from the overall confidence that this mode of action is also operative in female mice.

Alternative MOAs for liver tumors were sufficiently investigated and ruled out**.** Further, the CARC concluded that the proposed MOA evaluated was relevant to humans since the mechanistic data was insufficient to reasonably refute the relevance of the proposed MOA to humans.

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT OF THE EVIDENCE

The CARC considered the following in its weight-of-evidence deliberation in assessing the carcinogenic potential of PBO.

Mice

Liver Tumors

- **The CARC determined that the combined hepatocellular adenomas and/or carcinomas, driven by adenomas, seen at ≥100 mg/kg/day in male mice are treatment related.** This was based on significant increasing trends in hepatocellular adenomas and combined adenomas and/or carcinomas, as well as significant differences in the pair-wise comparisons of the 100 and 300 mg/kg/day dose groups with the controls for hepatocellular adenomas and combined adenomas and/or carcinomas, all at $p<0.01$. For carcinomas, there was a significant increasing trend and significant difference in the pair-wise comparison of the 300 mg/kg/day dose group, both at $p<0.01$. The incidences of liver adenomas at 100 and 300 mg/kg/day were outside the historical control ranges of both the conducting laboratory and Charles River Laboratories. The incidence of liver carcinomas at 300 mg/kg/day was just outside the historical control ranges from both the conducting laboratory and Charles River Laboratories. Supporting pre-neoplastic lesions (liver hyperplasia and eosinophilic foci) were seen at 300 mg/kg/day at study termination. While the CARC relied primarily on the tumor data as reported in MRID 42903701, it also considered a 1995 pathology re-evaluation of the liver tumors. The overall conclusion, however, was the same--that the combined liver tumors, driven by adenomas, were treatment related.

- **The CARC determined that the liver adenomas seen at 300 mg/kg/day (highest dose tested) in female CD-1 mice were treatment related.** Female mice had a significant increasing trend, and a significant difference in the pair-wise comparison of the 300 mg/kg/day dose group with the controls, for adenomas, both at $p<0.01$. There were no liver carcinomas reported in the females. The incidence of liver adenomas at 300 mg/kg/day was outside the historical control ranges for both the conducting laboratory, Bushy Run Research Center, and Charles River. Supporting pre-neoplastic lesions (liver hyperplasia, and eosinophilic foci) were seen at 300 mg/kg/day at study termination.

- The CARC concluded that dosing in the mouse carcinogenicity study was adequate and not excessive to assess carcinogenicity. This was based on liver histopathology (eosinophilic foci and hyperplasia) seen at 300 mg/kg/day in males and at ≥100 mg/kg/day in females. Liver hypertrophy was also seen in males at ≥100 mg/kg/day and in females at 300 mg/kg/day. Increased liver weights were seen in both sexes at ≥100 mg/kg/day. Survival was not affected.

Rats

- **The CARC concluded that the combined thyroid follicular cell adenomas and/or carcinomas seen in male rats and the thyroid follicular cell adenomas seen in female rats are not treatment related at doses that were considered to be adequate, and not excessive, for evaluating carcinogenicity.** For males, this was based on a significant increasing trend only in thyroid follicular cell combined adenomas and/or carcinomas at $p < 0.05$, but no significant differences seen in the pair-wise comparisons of any dosed group with the controls for thyroid follicular cell adenomas, carcinomas or combined. In addition, there was a lack of a dose response at the lower doses. The incidences of thyroid follicular cell adenomas or carcinomas alone at 500 mg/kg/day were within the Charles River historical control ranges. The incidences of thyroid follicular cell combined adenomas and/or carcinomas at 500 mg/kg/day are just above the conducting laboratory historical control range. For females, this was based on a significant increasing trend only in thyroid follicular cell adenomas at $p<0.05$, but no significant differences in the pair-wise comparisons of the dosed groups with the controls. There were no thyroid follicular cell carcinomas reported in the female rats. There was a lack of a strong dose response and very few tumors were seen overall. The incidences of adenomas were within the conducting laboratory and Charles River Laboratories historical control ranges.

- **The CARC concluded that the liver tumors seen in male and female rats were not treatment related.** For males, this was based on significant increasing trends only in adenomas at $p<0.01$ and combined adenomas and/or carcinomas at $p<0.05$, but no significant differences in the pair-wise comparisons of adenomas, carcinomas or combined compared to controls at any dose. For females, the incidences of liver carcinomas were not biologically or statistically significant at any dose. There were no liver adenomas reported for females. For both sexes, the incidences of liver adenomas and/or carcinomas were within or just outside the in-house and Charles River historical control ranges.

- Dosing was considered adequate, and not excessive, based on thyroid histopathology (thyroid follicular cell hyperplasia at ≥100 mg/kg/day in females), decreased body weight (both sexes) at 500 mg/kg/day, increased liver weight, clinical pathology (increased cholesterol) and macroscopic (focal mixed cells) and microscopic pathology (hypertrophy) findings in the liver at 500 mg/kg/day (both sexes)). The mortality noted in males at the very end of the study did not compromise the integrity of the study overall and still allowed for valid statistical comparisons of tumors.

Mutagenicity

The CARC concluded that the overall weight of the evidence did not show a mutagenic concern of PBO based on a battery of genotoxicity assays.

Structure Activity Relationship

There is limited SAR support which identified other synergists, including MGK-264, piperonyl sulfoxide, and safrole, with structural similarities of <80%, which were also potentially carcinogenic.

Mode of Action

The registrant proposed a non-genotoxic MOA for liver tumors in male and female mice. In the proposed MOA for liver tumors in mice, CAR activation in the liver leads to CYP2B induction (key event 1), subsequent cell proliferation (key event 2), increased liver weight and liver hypertrophy (associative events 1 and 2), clonal expansion leading to altered liver foci (key event 3), and ultimately liver tumors (adverse outcome).

The registrant proposed a mitogenic MOA involving constitutive androstane receptor (CAR) activation for liver tumors observed in male and female mice after PBO treatment. It is noted that the MOA data were conducted in male mice only since the males were more susceptible to PBO-induced liver tumor formation than female mice. The CARC considered the data generated using just one sex representative of the response in both sexes and does not detract from the overall mode of action. The CARC concluded that the submitted data adequately support the proposed MOA based on the following considerations.

Key Event #1: CAR Activation and Enzyme Induction in the Liver. The CARC concluded that key event 1 is supported. Evidence for CAR activation was demonstrated in male mice in mechanistic studies which demonstrated significant and dose-related increases in Cyp2b10 mRNA levels, PROD enzyme activity, and P-450 protein levels seen after 14 days of exposure to 30-300 mg/kg/day PBO, including robust increases at the tumorigenic doses of ≥100 mg/kg/day. This key event was demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., C57BL/6J wildtype and C57BL/6J CAR and PXR knockout, CAR KO/PXR KO), where little or no CAR activity (gene or protein expression of Cyp2b10) was seen at the carcinogenic dose levels in the KO mice. Furthermore, the Cyp2b/CAR-associated gene expression and protein data as well as the increased cell proliferation data from these MOA experiments in mice define a very specific PBO CAR MOA as the dominant activator, while simultaneously acknowledging some crosstalk with other liver nuclear receptors. However, a primary role of other nuclear receptor-mediated MOAs for rodent hepatic carcinogens such as pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR-α), or aromatic hydrocarbon-responsive receptor (AhR) agonism is ruled out.

Key Event #2: Hepatocyte Proliferation. The CARC agreed that key event #2 (increased cell proliferation) is supported. A burst of proliferation, measured by a dose related increase in induction of replicative DNA synthesis was seen only at 1 week of PBO treatment of male CD-1 mice over 7, 14 and/or 36 days at dose levels 30-300 mg/kg/day, including at the tumorigenic dose levels, with statistically significant increases seen at 300 mg/kg/day. This key event was demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., C57BL/6J wildtype and C57BL/6J CAR and PXR knockout, CAR KO/PXR KO), where reduced CAR activity (increased labeling index) was seen at 300 mg/kg/day in the double KO mice.

Associative Events #1 (increased liver weight) and #2 (increased liver hypertrophy): The CARC agreed that Associative Events #1 and #2 are adequately supported by the data. Activation of CAR was evaluated indirectly following PBO exposure through an increase in liver weight and liver hypertrophy. At 300 mg/kg/day, robust increases were seen in liver weight after 7, 14, and 42 days exposure to PBO in CD-1 male mice. Dose related significantly increased liver weights were seen following 78 weeks exposure to PBO at 100 and 300 mg/kg/day in both male and female CD-1 mice. Increased liver

hypertrophy was seen at 7 and 14 days at 300 mg/kg/day PBO in male CD-1 mice. Increased liver hypertrophy was observed in CD-1 males at ≥100 mg/kg/day and in CD-1 females at 300 mg/kg/day following 78-weeks exposure to PBO. Both of the associative events were demonstrated to be directly tied to the activity of the CAR nuclear receptor by the use of genetically modified mouse models (i.e., C57BL/6J wildtype and C57BL/6J CAR and PXR knockout, CAR KO/PXR KO), where increased liver weight and hypertrophy were seen at 300 mg/kg/day in the wildtype mice but not in the double KO mice.

Key Event #3: Clonal Expansion Leading to Altered Hepatic Foci. The CARC noted an inconsistency in the proposed MOA with key event #3 (clonal event leading to altered liver foci). In the 78-week mouse study, a slight (not statistically significant) increase in liver foci was observed at 300 mg/kg/day in both sexes, with no increase in males at 100 mg/kg/day. While key event #3 remains an inconsistency, increases in altered foci are not always able to be observed, and a documented increase in the number of altered foci in rodent livers may not be identified in a particular study because of the timing of sacrifice(s) and the intrinsic characteristics of the chemical of interest.

Key Event #4 (liver tumor formation): The CARC determined that liver tumors, driven by adenomas, were treatment related at ≥100 mg/kg/day in males and 300 mg/kg/day in females in a 78-week dietary study in mice.

Based on the evidence presented, the CARC concluded that there is strong concordance between key (and associative) events and the dose levels that produce tumors. The CARC also concluded that the key and associative events occur in a logical, time-dependent manner consistent with the proposed CAR MOA.

The CARC determined that alternative MOAs (*i.e.,* genotoxicity, cytotoxicity, hormonal perturbation, immunosuppression, infections, metal overload, increased apoptosis, and a primary role of mitogenesis induced by crosstalk with other nuclear receptors such as hepatic AhR, PXR, and hepatic PPARα) have been adequately ruled out as the primary operative MOA leading to tumor formation following PBO exposure.

The CARC concluded that the proposed MOA evaluated is relevant to humans.

Overall, the CARC concluded that the weight of evidence supports a CAR-mediated mitogenic MOA for PBO-related liver tumors in male and female mice.

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's *Guidelines for Carcinogen Risk Assessment* (March, 2005), the CARC classified PBO as “Not likely to be carcinogenic to humans at doses that do not induce cellular proliferation in the liver.” This classification was based on the following WOE considerations:

1. Treatment-related increases in combined liver adenomas and/or carcinomas, driven by adenomas, were observed in male CD-1 mice at 100 and 300 mg/kg/day and treatment related liver adenomas were observed in female CD-1 mice at 300 mg/kg/day.

2. Both the liver tumors and thyroid follicular cell tumors seen in male and female Sprague Dawley rats at 500 mg/kg/day were not considered to be treatment-related.
3. The mechanistic data support the proposed mitogenic MOA involving CAR activation for liver tumors observed in male and female mice after PBO treatment.
4. Based on the total weight of evidence of the available data, there is no significant concern for the mutagenicity of PBO.
5. There is limited SAR support which identified other synergists, including MGK-264, piperonyl sulfoxide, and safrole, with structural similarities of <80%, which were also potentially carcinogenic.

VII. QUANTIFICATION OF CARCINOGENIC POTENTIAL

Based on this cancer classification, quantification of cancer risk is not required. A non-linear approach (i.e., Reference Dose (RfD)) would adequately account for all the chronic toxicity, including carcinogenicity, that could result from exposure to PBO. The RfD should be protective of the dose (30 mg/kg/day) which induced hepatocellular proliferation in mice.

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Appendix A: Literature Search for PBO Cancer Reassessment

As part of this review, a broad survey of the literature was conducted in 2023 to identify studies that report toxicity following exposure to PBO or elucidate potential mechanisms of carcinogenicity via exposure routes relevant to human health pesticide risk assessment not accounted for in the Agency's PBO toxicology database. The search strategy employed terms for the name of the chemical plus any common synonyms, mechanistic terms for carcinogenicity, and terms relevant to the evaluated mechanisms. Two search strings (Table A1) were constructed using the National Toxicology Program Handbook for Preparing Report on Carcinogens Monographs (2015) search strings for cancer mechanisms as a template and expanding with various terms relevant to the specific MOAs in order to be as comprehensive as possible. Due to the low number of hits, a third search string containing only the chemical name and synonyms was used to ensure all relevant literature was captured.

The search strategy returned 185 studies from the literature. Following title/abstract and/or full text screening, 4 studies were identified as containing potentially relevant information (either quantitative or qualitative) for consideration in PBO cancer reclassification. Two primary literature reviews described potential mechanisms of carcinogenicity for PBO to support the liver tumor MOA, and two mutagenicity studies provided additional support for PBO being non-mutagenic. See list of citations in embedded excel files.


PBO%20search_One_164-hits_headers.xl


PBO%20search_two_21%20hits_headers

Mechanistic Articles

Sakamoto, Y., Inoue, K., Takahashi, M., *et al.* (2013) Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. *Toxicol. Pathol.* 41, pp. 1078-1092. MRID 52376003.

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All searches conducted in PubMed on 09/27/2023 at 10 am EST. Review of literature conducted following the OPP/HED guidance on "Procedure for Conducting Open Literature Reviews for Use in OPP's Human Health Risk Assessments" (2023).

Table A1. Search Criteria for Screening-level Literature Search.

Search 1: *Piperonyl Butoxide (synonyms + PBO) + NIH Keywords for Cancer MOA*

Search Details:
U.S. National Library of Medicine (NLM). Do not reply directly to this message
Sent On: Wed Jun 14 07:29:47 2023

Date and Time of Search: 07/31/2023; 03:25 pm

Custom Search TWO Details:
(piperonyl butoxide OR PBO) AND ((((("etiology"[sh] OR "Causality"[mh] OR "tumor markers, biological"[mh] OR "oncogene fusion"[mh] OR "tumor necrosis factors"[mh] OR adverse-outcome-pathway*[tiab] OR biological-marker[tiab] OR biological-markers[tiab] OR biomarkers[tiab] OR biomarker[tiab] OR Biotransformation[tiab] OR etiology[tiab] OR Key Event*[tiab] OR Mechanism-of-action[tiab] OR Mechanisms-of-action[tiab] OR Mode-of-action[tiab] OR modes-of-action[tiab] OR Molecular-Initiating-Event*[tiab] OR neoplastic-cell-transform*[tiab] OR Phosphorylation[tiab] OR Toxicity-Pathway*[tiab] OR toxicokinetic*[tiab] OR toxic-pathway*[tiab]) AND (Cancer[sb])) OR (tumor-inhibit*[tiab] OR tumor-promot*[tiab] OR tumour-inhibit*[tiab] OR tumour-promot*[tiab] OR Oncogenes[tiab] OR Oncogenesis[tiab] OR Oncogenic[tiab] OR pathogenesis[tiab])))

PubMed hits: 21
Number of Swift Articles: 19 for Animal
Number of Swift Articles: 6 for Human
Number of Swift Articles: 0 for No Tag

Search 2: *Chemical (synonyms) + NIH Keywords for Cancer MOA + Target Organs/Effects*

Search Details:
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Sent On: Wed Jun 14 07:27:34 2023

Search: (piperonyl butoxide OR PBO) AND ((("angiogenesis inducing agents"[mh] OR "myelodysplastic-myeloproliferative diseases"[mh] OR "neoplasms"[mh] OR "carcinogenicity tests"[mh] OR "carcinogens"[mh] OR (sentinel-lymph-node[tiab] NOT biopsy[tiab]) OR (ASCO[tiab] NOT fungi[tiab]) OR (WAGR[tiab] AND syndrome[tiab]) OR 5q-syndrome[tiab] OR leukostasis[tiab])) OR ((acanthoma[tiab] OR acanthomas[tiab] OR acrochordon[tiab] OR acrochordons[tiab] OR acrospiroma[tiab] OR acrospiromas[tiab] OR adamantinoma[tiab] OR adamantinomas[tiab] OR adenoacanthoma[tiab] OR adenoacanthomas[tiab] OR adenoameloblastoma[tiab] OR adenoameloblastomas[tiab] OR adenocanthoma[tiab] OR adenocanthomas[tiab] OR adenocarcinoma[tiab] OR adenocarcinomas[tiab] OR adenofibroma[tiab] OR adenofibromas[tiab] OR adenolipoma[tiab] OR adenolipomas[tiab] OR adenolymphoma[tiab] OR adenolymphomas[tiab] OR adenoma[tiab] OR adenomas[tiab] OR adenomatosis[tiab] OR adenomatous[tiab] OR adenomyoepithelioma[tiab] OR adenomyoepitheliomas[tiab] OR adenomyoma[tiab] OR adenomyomas[tiab] OR adenosarcoma[tiab] OR adenosarcomas[tiab] OR adenosis[tiab] OR aesthesioneuroblastoma[tiab] OR aesthesioneuroblastomas[tiab] OR ameloblastoma[tiab] OR ameloblastomas[tiab] OR amyloidoses[tiab] OR amyloidosis[tiab] OR anaplasia[tiab] OR androblastoma[tiab] OR androblastomas[tiab] OR angioblastoma[tiab] OR angioblastomas[tiab] OR angioendothelioma[tiab] OR angioendotheliomas[tiab] OR angioendotheliomatosis[tiab] OR angiofibroma[tiab] OR angiofibromas[tiab] OR angiofibrosarcoma[tiab] OR angiokeratoma[tiab] OR angiokeratomas[tiab] OR angioleiomyoma[tiab] OR angioleiomyomas[tiab] OR angiolipoma[tiab] OR angiolipomas[tiab] OR angioma[tiab] OR angiomas[tiab] OR angiomatosis[tiab] OR angiomyolipoma[tiab] OR angiomyolipomas[tiab] OR angiomyoma[tiab] OR angiomyomas[tiab] OR angiomyxoma[tiab] OR angiomyxomas[tiab] OR angioreticuloma[tiab] OR

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