β-Myrcene

Should Not be Listed as a Proposition 65 Carcinogen

Pursuant to the Authoritative Bodies Listing Process

Comments of

Flavor and Extract Manufacturers Association

International Fragrance Association, North America

Juice Products Association

Renewable Citrus Products Association

Submitted to the
Office of Environmental Health Hazard Assessment

by

F. Jay Murray, Ph.D.
Gary M. Roberts

April 10, 2012

Table of Contents

I. Introduction 1

II. β-Myrcene rat data should not form the basis for cancer hazard identification 2

III. β-Myrcene mouse data should not form the basis for cancer hazard identification... 7

A. The male mouse data demonstrated an increase in liver tumors in B6C3F1 mice, a strain with a high background rate of and high degree of susceptibility to liver tumors 7

B. Mouse liver tumors require additional expert analysis in cancer hazard identification because of serious questions concerning sufficiency of such evidence 8

C. B6C3F1 mouse liver tumors are not a reliable indicator of carcinogenic hazard. 12

IV. β-Myrcene is not genotoxic 14

V. β-Myrcene should not be listed because the NTP has not found "sufficient evidence" of carcinogenicity in animals. 15

VI. Conclusion 21

I. Introduction

β-Myrcene should not be listed as a carcinogen because both the mouse and rat data upon which the NTP identified carcinogenic activity under the conditions of NTP's study are of dubious relevance to cancer hazard identification. The NTP did not perform an analysis that extended beyond the conditions of its Technical Report data to evaluate whether β-myrcene causes cancer in animals. Thus, the Flavor and Extract Manufacturers Association, the International Fragrance Association, North America, the Juice Products Association and the Renewable Citrus Products Association ("Associations") oppose listing β-myrcene as a Proposition 65 carcinogen. In particular, the Associations oppose listing β-myrcene as a carcinogen through the authoritative bodies process. If California wishes to proceed with a listing evaluation of β-myrcene, it should do so by referring review of β-myrcene to the Carcinogen Identification Committee (CIC).

The data suggesting carcinogenic activity for β-myrcene come from NTP Technical Report No. 557 (TR-557).[1] There are only two findings of "clear evidence of carcinogenic activity": kidney tumors in male rats and liver tumors in male mice. Both involve tumors that occur at a high background incidence, are suspect due to genetic predisposition and are of doubtful relevance for cancer hazard identification. In both cases, these findings occurred in studies where there were only two dose levels available to evaluate carcinogenic potential because NTP decided not to present the histopathological data at the high dose due to excessive mortality. No other data exist

[1] National Toxicology Program (NTP). 2010. NTP Technical Report on the Toxicology and Carcinogenesis Studies of β-Myrcene (CASRN 123-35-3) in F344/N Rats and B6C3F1 Mice. (NTP TR-557). National Toxicology Program, Research Triangle Park, NC.

that support a finding of sufficient evidence of carcinogenicity. And, β-myrcene was not genotoxic in a battery of tests conducted by NTP.

The Associations request that OEHHA either proceed no further with the listing of β-myrcene or that the CIC review the data for β-myrcene before OEHHA takes any further regulatory action. The Associations also request the opportunity to meet with OEHHA to further discuss the legal and scientific issues raised by its evaluation of β-myrcene.

II. β-Myrcene rat data should not form the basis for cancer hazard identification

The NTP rat data shows an increase in kidney tumors in male rats, a species- and sex-specific response unique to male rats and of doubtful relevance to cancer hazard identification. In female rats, there was no significant increase in any tumor at any dose level, and NTP categorized the evidence of carcinogenic activity as "equivocal." Thus, the rat data is not a sufficient basis on which to move forward with an authoritative body listing.

In the NTP bioassay, male F344/N rats were administered 0, 250, 500, and 1000 mg/kg/day of β-myrcene by gavage for 5 days/week. The evaluation of the potential carcinogenicity of β-myrcene was limited to the low- and mid-dose levels because the high dose caused such excessive mortality that NTP decided not to even present the histopathological data for the high-dose group of male rats:

> "Due to the early mortality in the 1 g/kg male rats, data from this group are not presented in this section."[2]

At the middle and low doses, a statistically significant increase in renal tubule adenomas and combined adenomas and carcinomas was observed among the male rats. No statistically significant increase in renal tubule carcinomas alone was seen at either dose level. In addition, a clear dose-response relationship was not evident for any renal tubule neoplasm since there was very little difference between the results at the low and middle doses. For example, the incidence of renal tubule adenomas (single and step sections) was 0% (0/50), 24% (12/50), and 26% (13/50) at 0, 250 and 500 mg/kg/day, respectively. The incidence of renal tubule carcinomas (single and step sections was 0% (0/50), 6% (3/50), and 2% (1/50) at 0, 250 and 500 mg/kg/day, respectively.

For male rats, the NTP Technical Report concluded: "Under the conditions of these 2-year gavage studies, there was clear evidence of carcinogenic activity of β-myrcene in male F344/N rats based on increased incidences of renal tubule neoplasms." However, it should be recognized that NTP qualified its statements regarding the level of carcinogenic activity with the phrase "under the conditions of these 2-year gavage studies." Importantly, NTP did not indicate whether it considered these results to be relevant for purposes of hazard identification.

Male kidney tumors are of questionable relevance for cancer hazard identification since they appear to be a species- and sex-specific response that is unique to the male

[2] National Toxicology Program (NTP). 2010. NTP Technical Report on the Toxicology and Carcinogenesis Studies of β-Myrcene (CASRN 123-35-3) in F344/N Rats and B6C3F1 Mice. NTP TR-557. NIH Publication No. National Toxicology Program, Research Triangle Park, NC, p. 39.

rat.[3,4] As expected, in female rats, there was no statistically significant increase in renal tubule adenomas or carcinomas (or any other tumor) at any dose level of β-myrcene. NTP decided there was "equivocal evidence of carcinogenic activity" in female rats based on a slight (not statistically significant) increase in the incidence of benign kidney tumors compared to historical controls.

Many chemicals have been found to cause kidney tumors in male rats, but not in female rats or male or female mice.[5] Examples include *d*-limonene, 1,4-dichlorobenzene and isophorone. This phenomenon reflects the sensitivity of the male rat kidney to chronic progressive nephropathy. And, in the NTP bioassay of β-myrcene, chronic progressive nephropathy was pronounced in male rats.

NTP discussed the possible relationship between the appearance of kidney tumors and the non-neoplastic lesions found in the kidneys:

> "In the 2-year rat study, there was clear evidence of carcinogenic activity of β-myrcene in male rats based on the increased incidences of renal tubule adenoma or carcinoma. . . . β-myrcene administration also resulted in increased incidence and/or severity of a number of non-neoplastic renal lesions, including nephrosis and exacerbations of CPN in both sexes, and papillary mineralization in the males. The papillary mineralization had a linear appearance and was

---

[3] Travlos GS, Hard GC, Betz LJ, Kissling GE (2012) Chronic Progressive Nephropathy in Male F344 Rats in 90-Day Toxicity Studies, Its Occurrence and Association with Renal Tubule Tumors in Subsequent 2-Year Bioassays. Toxicol Pathol 40: 473-481.

[4] Swenberg JA, Lehman-McKeeman LD (1999). α_2–Urinary globulin-associated nephropathy as a mechanism of renal tubule cell carcinogenesis in male rats. In Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis (CapenC. C., DybingE., RiceJ. M., WilbournJ. D., eds.), pp. 95–118. IARC Publications No. 147, International Agency for Research on Cancer, Lyon.

[5] Lock EA, Hard GC (2004) Chemically induced renal tubule tumors in the laboratory rat and mouse of the NCI/NTP database and categorization of renal carcinogens based on mechanistic information. Crit Rev Toxicol 34(3):211-299.

found in the loops of Henle in the medulla. This type of mineralization, which is considered a chronic manifestation of α2u-globulin nephropathy, was also seen in NTP chronic studies of the structurally related compound *d*-limonene (NTP, 1990; Hard et al., 1993).

"Nephrosis was unique lesion in the 2-year study of β-myrcene in rats and was more severe in males than in females. The pathogenesis of this lesion is unknown, but the co-localization of this lesion with the renal tubule necrosis in the outer stripe of the outer medulla (in the 3-month study) and the proliferative nature of the nephrosis (as evidenced by the karyomegaly and tubule hyperplasia) suggest that it is an unusual response to repeated renal tubule epithelial cell injury, primarily in the P3 segment of the proximal tubules. Whether or not this unusual regenerative response could lead ultimately to neoplasia , either directly or through exacerbation of CPN, is not clear. Nephrosis was not seen in the *d*-limonene studies, nor was renal tubule necrosis seen in the outer stripe of the outer medulla (NTP, 1990).

"The mechanism of β-myrcene -induced renal carcinogenesis in male . . . rats is not clear. The observation of α2u-globulin nephropathy and linear papillary mineralization in male rats suggests this syndrome as one potential mechanism of carcinogenesis. However, several lines of evidence suggest this syndrome as one potential mechanism of carcinogenesis. However, several lines of evidence suggest that β-myrcene might cause nephrotoxicity by a mechanism other than, or in addition to, α2u-globulin nephropathy. The incidence and severity of linear papillary mineralization were greatest in the 0.25 mg/kg males but slightly deceased in the 0.5 mg/kg males; this response is consistent with the decrease in the incidences of hyaline droplet accumulation seen in the 3-month study. Additionally, there were dose-related increases in the incidence and severity of CPN and nephrosis in both the male and female rats. The presence of renal neoplasms in female rats also suggests a mechanism of carcinogenesis that may be related to the nephrosis and is distinct from the α2u-globulin mechanism."[6]

Although NTP discussed the relationship between the kidney toxicity and kidney tumors observed in the male rats under the conditions of the β-myrcene study, NTP never indicated whether it considered these results to be relevant for purposes of hazard identification. In fact, NTP's discussion is further evidence that expert judgment would be required to determine the relevance of the male kidney tumors beyond the conditions of the study for purposes of cancer hazard identification. Also, NTP's

[6] National Toxicology Program (NTP). 2010. NTP Technical Report on the Toxicology and Carcinogenesis Studies of β-Myrcene (CASRN 123-35-3) in F344/N Rats and B6C3F1 Mice. NTP TR 557. NIH Publication No. National Toxicology Program, Research Triangle Park, NC, p. 62.

mention of the "presence of renal neoplasms in female rats" in the context of exploring the mechanism of action should not be over interpreted. The presence of a few tumors in the female rats was not statistically significant and is not described by NTP as biologically meaningful. There was no statistically significant increase in any renal neoplasms in female rats and NTP concluded the evidence of carcinogenic activity in female rats was "equivocal," as discussed in the next section. Thus, there is every possibility that the significant increase in kidney tumors in male rats is explained by the α2u-globulin mechanism, a mechanism known to be species- and sex-specific. The renal pathology reported in male F344/N rats in the NTP bioassay of β-myrcene is similar to that of other substances tested in NTP bioassays (e.g., *d*-limonene, pinene) that have been associated with kidney tumors. Although high doses of these substances have been shown to cause kidney tumors in male rats, these findings are widely considered irrelevant to humans because the tumors are unique to the male rat. In fact, *d*-limonene is considered by some researchers to be a potential chemopreventive agent.[7] IARC classifies *d*-limonene as a Group 3 carcinogen: *not classifiable as to its carcinogenicity to humans.*[8]

[7] Tsuda, H, Ohshima Y,; Nomoto H, Fujita K, Matsuda E, Iigo M, Takasuka N, Moore M (2004) Cancer Prevention by Natural Compounds. Drug Metabolism and Pharmacokinetics 19 (4): 245–63.

[8] IARC (1999) Volume 73. Some Chemicals that Cause Tumors of the Kidney or Urinary Bladder in Rodents and Some Other Substances.

III. β-Myrcene mouse data should not form the basis for cancer hazard identification

A. The male mouse data demonstrated an increase in liver tumors in B6C3F1 mice, a strain with a high background rate of and high degree of susceptibility to liver tumors

The NTP bioassay of β-myrcene states: "There was *clear evidence of carcinogenic activity* of β-myrcene in male B6C3F1 mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma."[9] These statistically significant increases were seen at the mid-dose level of 500 mg/kg/day. At the low dose (250 mg/kg/day), the only statistically significant response was an increase in hepatocellular adenoma, a benign liver tumor.

The high dose level (1000 mg/kg/day), which was not properly selected, produced excessive mortality. There was a statistically significant decrease in survival among high dose males compared to controls, and the cause of deaths was uncertain.[10] As in the case of the high dose male rats, NTP decided not to even present the histopathological data for the high-dose group of male mice due to high early mortality (i.e. acute toxicity). [11]

The evaluation of the dose-response relationship is complicated by the fact that there were only two exposed groups assessed for potential carcinogenicity since there were no histopathological data at the high-dose due to excessive mortality. The background incidence of hepatocellular tumors among the control males in this study was extremely high. For example, the incidence of liver adenomas and carcinomas

---

[9] TR-557 at 9.

[10] TR-557 at 50.

[11] *Id.*

among the control male mice in the β-myrcene bioassay was 66%. In other words, approximately 2/3 of the control males had liver tumors. In effect, β-myrcene caused an increase in the incidence of liver tumors that most of the mice would have even if they weren't exposed to β-myrcene. This high background rate is a strong indicator that this strain is highly susceptible to liver tumors. Further, mouse liver tumors are of dubious relevance to cancer hazard assessment, as discussed in subsequent sections of this submission.

B. Mouse liver tumors require additional expert analysis in cancer hazard identification because of serious questions concerning sufficiency of such evidence

The predictive value of mouse hepatocellular tumors with respect to cancer risk has been repeatedly challenged.[12,13] This is in part due to the fact that hepatocellular carcinoma in humans, particularly chemically-induced, is rare. In humans, the major risk factors associated with liver tumors are viral hepatitis, excessive alcohol consumption, and exposure to aflatoxin, in most cases accompanied by liver cirrhosis.

The European Food Safety Authority (EFSA) has concluded that "hepatic tumors in mice are generally considered as irrelevant for human risk assessment" in mouse dietary administration study.[14] Gavage administration, which replicates actual

---

[12] Velazquez SF, Schoeny R, Rice GE, Cogliano VJ (1996). Cancer risk assessment: historical perspectives, current issues, and future directions. Drug Chem Toxicol 19(3):161-185.

[13] Carmichael NG, Enzmann H, Pate I, Waechter F (1997). The significance of mouse liver tumor formation for carcinogenic risk assessment: results and conclusions from a survey of ten years of testing by the agrochemical industry. Environ Health Perspect 105(11):1196-1203.

[14] EFSA (2011). European Food Safety Authority; EFSA Statement on the scientific evaluation of two studies related to the safety of artificial sweeteners (question no EFSA-Q-2011-00064, approved on 25 February 2011 by European Food Safety Authority). EFSA J 9(2):2089 [16 pp.]. doi:10.2903/j.efsa.2011.2089. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/2089.htm.

exposures even less, also should be considered irrelevant. Beginning in 2000, the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) of Australia has concluded that the liver tumors observed in B6C3F1 mice after prolonged exposure to a range of chemicals (e.g., p-dichlorobenzene) are considered to be irrelevant to humans.[15] During these evaluations NICNAS has emphasized that the high natural spontaneous incidence of liver tumors in this strain and sex of mice significantly affects the ability to interpret the results.

Induction of hepatocellular tumors in mice by non-genotoxic compounds can be considered as irrelevant for human risk assessment.[16,17] In their evaluation of the mode of action with respect to the relevance of rodent liver tumors to human cancer risk, Holsapple *et al*. (2006) concluded that in the case of chemicals displaying a phenobarbital-like P450 inducing mode of action, the observed hepatocarcinogenicity in rodents is not relevant to humans. Indeed, clinical use for over 80 years of phenobarbital, a known enzyme inducer in the rodent liver, has not been associated with an increased risk of tumor formation in the liver or any other organ in humans.[18] It is generally well accepted that male and female B6C3F1 mouse liver tumors that arise

---

[15] Commonwealth of Australia, 2000. National Industrial Chemical Notification and Assessment Scheme (NICNAS), December 2000, Commonwealth of Australia, 134 pp.

[16] Holsapple, M.P., Pitot, H.C., Cohen, S.M., Boobis, A.R., Klaunig, J.E., Pastoor, T., Dellarco, V.L., Dragan, Y.P., 2006. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89, 51–56.

[17] Billington R, Lewis R.W, Mehta J.M, Dewhurst I (2010). The mouse carcinogenicity study is no longer a scientifically justifiable core data requirement for the safety assessment of pesticides Crit Rev Toxicol 40(1):35-49.

[18] McClain RM (1990). Mouse liver tumors and microsomal enzyme-inducing drugs: experimental and clinical perspectives with phenobarbital. In: Stevenson DE, Popp JA, Ward JM, McClain RM, Slaga TJ, Pitot HC, editors. *Mouse Liver Carcinogenesis: Mechanisms and Species Comparisons*. Symposium, Nov. 30-Dec. 3, 1988, Austin, Texas. (Progress in Clinical and Biological Research, vol 331). New York (NY): Wiley-Liss, pp. 345-365. Cited In: Carmichael et al., 1997 [Ref. #34].

in 2-year bioassays with various agents are an indirect result of dose-related chronic toxicity and resulting cellular proliferation. In the absence of this chronic toxicity, these tumors are not considered to represent a cancer hazard for humans (Cohen et al., 2004).[20]

It appears that, in at least one case, NTP has called into question the relevance of mouse liver tumors for purposes of hazard identification. In an NTP bioassay (NTP TR-190), p-nitrosodiphenylamine caused "positive" findings of liver tumors in male mice and male rats:

> "Under the conditions of this bioassay, p-nitrosodiphenylamine was carcinogenic when administered in the diet to male B6C3F1 mice, causing hepatocellular carcinomas. The chemical was also carcinogenic in male Fisher 344 rats, causing liver neoplasms. No evidence was provided for the carcinogenicity of p-nitrosodiphenylamine in female B6C3F1 mice or in female Fisher 344 rats."[21]

In 1989, NTP identified p-nitrosodiphenylamine as a carcinogen in its Fifth Annual Report on Carcinogens. Subsequently, NTP delisted p-nitrosodiphenylamine for insufficient evidence of carcinogenicity in its Sixth Annual Report on Carcinogens, which was published in 1991. We are currently searching for a copy of the Sixth Annual Report on Carcinogens to further investigate the reason for delisting this substance. But, based on the results of the NTP bioassay, it is clear that the only reason

---

[20] Cohen S.M., Klaunig J., Meek M.E., Hill R.N., Pastoor T., Lehman-McKeeman L., Bucher J., Longfellow D.G., Seed J., Dellarco, V. 2004. Evaluating the human relevance of chemically induced animal tumors. *Toxicol. Sci.* 78: 181–186.

[21] National Toxicology Program (NTP). 1979. NTP Bioassay of p-nitrosodiphenylamine for possible carcinogenicity. (NTP TR-190). National Toxicology Program, Research Triangle Park, NC.

for initially listing it as a carcinogen was the rodent liver tumors, including the statistically significant increase in hepatocellular carcinoma in male mice. We also request that OEHHA hold open the record until we are able to obtain a copy of the Sixth Annual Report on Carcinogens and evaluate this information.

It appears that, in at least one case, NTP has called into question the relevance of mouse liver tumors for purposes of hazard identification. In an NTP bioassay (NTP TR-190), p-nitrosodiphenylamine caused "positive" findings of liver tumors in male mice and male rats:

> "Under the conditions of this bioassay, p-nitrosodiphenylamine was carcinogenic when administered in the diet to male B6C3F1 mice, causing hepatocellular carcinomas. The chemical was also carcinogenic in male Fisher 344 rats, causing liver neoplasms. No evidence was provided for the carcinogenicity of p-nitrosodiphenylamine in female B6C3F1 mice or in female Fisher 344 rats."

In 1989, NTP identified p-nitrosodiphenylamine as a carcinogen in its Fifth Annual Report on Carcinogens. Subsequently, NTP delisted p-nitrosodiphenylamine for insufficient evidence of carcinogenicity in its Sixth Annual Report on Carcinogens, which was published in 1991. We are currently searching for a copy of the Sixth Annual Report on Carcinogens to further investigate the reason for delisting this substance. But, based on the results of the NTP bioassay, it is clear that the only reason for initially listing it as a carcinogen was the rodent liver tumors, including the statistically significant increase in hepatocellular carcinoma in male mice. We also request that OEHHA hold

open the record until we are able to obtain a copy of the Sixth Annual Report on Carcinogens and evaluate this information.

C. B6C3F1 mouse liver tumors are not a reliable indicator of carcinogenic hazard

Although the NTP Technical Report states there is "clear evidence of carcinogenic activity" in male mice exposed to β-myrcene because of liver tumors, the Report did not address the issue of relevance of these mouse liver tumors to cancer hazard identification for other species. In fact, the high spontaneous incidence of hepatocellular tumors observed in B6C3F1 mice and the relevance of the development of these tumors in mice with regard to human cancer risk has been repeatedly questioned by scientists, including NTP scientists (Maronpot *et al.,* 1987; Velazquez *et al*., 1996).[22] The background incidence of liver tumors has been steadily rising over the past decade in the B6C3F1 mice used by the NTP in its cancer bioassays. Because of their high background rate of and high degree of susceptibility to liver tumors, B6C3F1 mice are not a reliable indicator of carcinogenic hazard for β-myrcene.

The background incidence of liver tumors in the B6C3F1 mice reported in NTP bioassays has historically been high, but in recent years, the background incidence of these tumors has significantly increased over even the historically high background rate. Prior to this recent dramatic change in the background incidence of liver tumors, the historical spontaneous incidence of liver neoplasms (combined hepatocellular adenoma and carcinomas) in control male $B6C3F_1$ mice in NTP bioassays was 32.4% with a

[22] Maronpot RR, Haseman JK, Boorman GA, Eustis SL, Rao GN, Huff JE (1987). Liver lesions in B6C3F1 mice: the National Toxicology Program, experience and position. Arch Toxicol Suppl 10:10-26.

range of 20-47%.[23] More recently, rates of combined hepatocellular adenoma and carcinomas in male B6C3F1 control mice exceeding 50% have been reported (e.g., 56% in the isoeugenol study (NTP, 2008), 58% in the pulegone study (NTP, 2011), and an astounding 66% in the β-myrcene study.[24,25,26] Thus, the incidence of combined hepatocellular adenoma and carcinoma in the control group of male B6C3F1 mice is outside the historical control range published by NTP in 2006, suggesting genetic drift in the mice used in the most recent NTP bioassays, including the bioassay of β-myrcene. The NTP has recognized the limitations of data pertaining to the development of liver tumors in the 2-year mouse bioassays, particularly in susceptible strains of mice (*e.g.*, B6C3F1), with respect to extrapolating the results to other species and has noted that alternative rodent strains are being examined to supplement rat studies.

---

[23] National Toxicology Program (NTP). 2006. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Benzophenone (CAS NO. 119-61-9) in F344/N Rats and B6C3F1 Mice. NTP TR 533. NIH Publication No. 06-4469. National Toxicology Program, Research Triangle Park, NC. http://ntp.niehs.nih.gov/

[24] National Toxicology Program (NTP). 2008. Draft Report: NTP Technical Report on the Toxicology and Carcinogenesis Studies of Isoeugenol (CAS NO. 97-54-1) in F344/N Rats and B6C3F1 Mice. NTP TR 551.

[25] National Toxicology Program (NTP). 2011. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Pulegone (CASRN 89-82-7) in F344/N Rats and B6C3F1 Mice. NTP TR 563. NIH Publication No. National Toxicology Program, Research Triangle Park, NC.

[26] National Toxicology Program (NTP). 2010. NTP Technical Report on the Toxicology and Carcinogenesis Studies of β-Myrcene (CASRN 123-35-3) in F344/N Rats and B6C3F1 Mice. NTP TR 557. NIH Publication No. National Toxicology Program, Research Triangle Park, NC.

IV. β–Myrcene is not genotoxic

β-Myrcene has not shown any evidence of genotoxicity. A battery of genotoxicity studies of β-myrcene was conducted by NTP.[27] No mutagenicity was observed in any of several strains of *Salmonella typhimurium* or *E. Coli* in two independent Ames test conducted with and without metabolic activation. In addition, β-myrcene was negative in a micronucleus test in male and female mice administered β-myrcene by gavage for three months.

Interestingly, two publications have reported that β-myrcene protects against known genotoxic substances. Investigators have studied the protective effects of monoterpenes, including β-myrcene, against t-butyl hydroperoxide-induced genotoxicity in reverse mutation assays with two strains of E. coli and with the comet assay in human hepatoma and lymphoid cells.[28] β-Myrcene had a substantial protective effect against oxidant-induced genotoxicity, which is predominately mediated by its radical scavenging activity. β-Myrcene also inhibited sister chromatid exchanges (SCE) caused by certain mutagens (i.e., cyclophosphamid, benzo[a]pyrene) in a dose-related manner, but it had no effect on SCE produced by aflatoxin B1 and DMBA; β-myrcene also reduced cyclophosphamid-induced SCE frequency in a hepatic tumor cell line.[29]

---

[27] National Toxicology Program (NTP). 2010. NTP Technical Report on the Toxicology and Carcinogenesis Studies of β-Myrcene (CASRN 123-35-3) in F344/N Rats and B6C3F1 Mice. NTP TR 557. NIH Publication No. National Toxicology Program, Research Triangle Park, NC, p. 9.

[28] Miti Ä, Culafi ÄD, Zegura B, Nikoli ÄB, Vukovi Ä,-Gaci ÄB, Knezevi Ä, Vukcevi ÄJ, Filipic M.(2009) Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem Toxicol 47(1):260-6. Epub 2008 Nov 18.

[29] Rascheisen C, Zamith H, Paumgartten FJ, Speit G (1991) Influence of beta-myrcene on sister-chromatid exchanges induced by mutagens in V79 and HTC cells. Mutat Res. 264(1):43-9.

V. β–Myrcene should not be listed because the NTP has not found "sufficient evidence" of carcinogenicity in animals.

OEHHA does not have the authority to list β-myrcene as a carcinogen because the NTP did not "conclude" that β-myrcene "causes cancer" in animals.[30] The "primary" Proposition 65 listing mechanism for candidate carcinogens is review by the "state's qualified experts," the Carcinogen Identification Committee ("CIC").[31] The "authoritative body" listing mechanism is supposed to be a shortcut, allowing listing without CIC review where an authoritative body has already done the work that the CIC would otherwise be required to do.[32] As relevant here, that mechanism is triggered only when a chemical has been "formally identified by an authoritative body as causing cancer" in a report which "concludes" that "[s]ufficient evidence of carcinogenicity exists from studies in experimental animals."[33] To constitute a "sufficient evidence" finding, the authoritative body's formal "report" must "conclude[]" that "studies in experimental animals indicate that there is an increased incidence of [cancer]."[34] OEHHA is not authorized to substantively evaluate the data on β-myrcene and conclude on its own that "sufficient evidence" of carcinogenicity exists. OEHHA's role is limited by regulation to the "ministerial" task of reviewing the authoritative body's formal reports and

---

[30] 27 CCR § 25306(a), (d)(1), and (e)(2).

[31] See Final Statement of Reasons ("FSR") for 27 CCR § 25306 (then 22 CCR § 12306) at 8.

[32] *Id*. at 5, 8.

[33] 27 CCR § 25306(a), (d)(1), (e)(2).

[34] 27 CCR § 25306(e)(2).

determining whether the authoritative body has, itself, issued a qualifying sufficient evidence "conclu[sion]."[35]

NTP has never "conclude[d]" that "sufficient evidence of carcinogenicity exists from studies in experimental animals" within the meaning of section 25306 for β-myrcene. Rather, the NTP expressed four separate and limited conclusions about carcinogenic activity in one strain of mice and one strain of rats *under the conditions of its experiment*. Moreover, as noted above, NTP commented that the effect seen was species specific, which further emphasizes the limited nature of the NTP statements and the absence of a "sufficient evidence" finding. NTP stated that "[t]he interpretative conclusions presented in NTP Technical Reports are based only on the results of these NTP studies. Extrapolation of these results to other species requires analyses beyond the intent of these reports."[36] NTP does evaluate chemicals for "sufficient evidence" of carcinogenicity in studies of experimental animals, applying a standard equivalent to section 25306(e)(2), but its current practice is to do so when evaluating chemicals for inclusion in its "Report on Carcinogens."

The plain language of section 25306 equates "sufficient evidence" with what "studies in experimental animals indicate" generally, and the regulatory history makes clear that this standard was intended to mirror the scientific consensus on sufficient evidence reflected in the language California borrowed directly from the EPA's 1986 Guidelines for Carcinogen Risk Assessment.[37] Those Guidelines require consideration

---

[35] FSR at 8.

[36] TR-577 at Foreword.

[37] 27 CCR § 25306(e)(2); (FSR at 15 (language drawn from EPA Guidelines).)

of all relevant studies, not just individual studies in isolation. OEMMA's interpretation would require it to list a chemical on the basis of a single positive study—even if other Technical Reports summarize equally valid, or more valid, data that calls into question the single positive study. NTP almost certainly would not agree in those circumstances that "studies in experimental animals indicate that there is an increased incidence of [cancer]," §25306(e)(2).

To the extent the language leaves any doubt, the regulatory history dispels it. It is undisputed that section 25306(e)'s "causing cancer" definition regarding animal evidence is the well known "sufficient evidence" test taken from the EPA's 1986 Guidelines for Carcinogen Risk Assessment, with the "same or substantially similar criteria" in use by the NTP, the authoritative body in question.[38] The FSR explains the regulation, repeatedly emphasizing that "sufficient evidence" is not a new standard for OEMMA scientists to administer or for industry scientists and observers to understand, but instead a standard already used by authoritative bodies to make their own cancer causing determinations. The FSR states:

> Subsection (e) provides that, for purposes of section 12306 [now 25306], the phrase "as causing cancer" means that either of two scientific criteria have been satisfied. Generally, the authoritative body may *rely* on either studies in humans or studies in animals. These criteria are consistent with the criteria the Panel presently uses in evaluating chemicals for listing. The Panel utilizes the EPA's Classification System for Categorizing Weight of Evidence for Carcinogens From Humans and Animal Studies (51 Fed. Reg. 33999 (Sept. 24, 1986)). The same, or substantially similar criteria have been adopted by many regulatory agencies and scientific organizations involved in hazard identification. The use of these criteria will ensure that *the standards applied by an authoritative body* are the same as or substantially similar to those used by the Panel to evaluate chemicals. (FSR at 15 (emphasis added)).

[38] *Compare* 27 CCR § 25306(e)(2) *with* 1986 EPA Cancer Guidelines at 33999.

* * * *

> It is not the intention of the Agency to *substitute its scientific judgment for that of the authoritative body*. The Agency's inquiry will be limited to whether the authoritative body *relied upon* scientific data in an amount sufficient *to conclude that the chemical causes cancer*. . . . *Because the body is considered authoritative, and the body utilizes the same or substantially the same criteria as set forth in section (e)*, it will be assumed that the data relied upon is scientifically valid. The Agency will look to determine whether the authoritative body relied upon animal or human data in an amount sufficient to satisfy the criteria. If so, the chemical will be proposed for listing.[39]

These FSR passages make it clear that the California Health and Welfare Agency, which wrote the regulation, expected the sufficient evidence standard would be "applied" by the authoritative body to "conclude that the chemical causes cancer." These two passages emphasize that the authoritative body is expected to exercise judgment in making the ultimate "causing cancer" conclusion according to substantially the same criteria as set forth in paragraph (e).

The 1986 EPA Guidelines for Carcinogen Risk Assessment provide that a "sufficient evidence" determination cannot be based on the results of individual animal studies considered in isolation, but must be based on a broader review of relevant data. EPA summarizes its standard as follows: "At various points in the above discussion, EPA has emphasized the need for an overall, balanced judgment of the totality of the available evidence."[40] The EPA Guidelines also state that "[r]eplicate negative studies

[39] FSR at 18 (emphasis added)).

[40] 33996 (left column).

that are essentially identical in all other respects to a positive study may indicate that the positive results are spurious."[41], [42]

Thus, the EPA cancer risk assessment guidelines, from which section (e)(2) was taken, require that all relevant "studies" be considered as a whole in making a "sufficient evidence" determination, whether based on animal or human data. Section (e)(2) was intended to implement the same standard. The regulation's copied language and the FSR make this abundantly clear. The NTP has not yet performed that overall analysis for β-myrcene, and thus its Technical Report does not contain a "sufficient evidence" determination required to support an authoritative body listing, or to render the CIC's consideration of β-myrcene unnecessary.

The NTP did not make a "sufficient evidence" finding with regard to β-myrcene. The Technical Report expresses carcinogenicity conclusions limited to "the conditions of these 2-year feed studies."[43] It does not render an overall conclusion about what "studies in experimental animals indicate, nor does it analyze the implications of the likely species-specific tumors for hazard identification" The Technical Report warns that its conclusions are not to be extrapolated "to other species, including characterization of hazards and risks to humans" because doing so would require "analyses beyond the intent" of the report.[44]

---

[41] 33995 (middle column).

[42] The EPA Guidelines also state expressly that the classification scheme "is not meant to be applied rigidly or mechanically," whenever there questionable positive data, but instead provides that "Results and conclusions concerning the agent, derived from different types of information, whether indicating positive or negative responses, are melded together…." *Id*. at 33996 (left column), 33994 (left column).

[43] TR-557 at 9.

[44] TR 577 at Foreword.

The Final Statement of Reasons expressly confirms, twice, that the "sufficient evidence" standard of section 25306(e) is meant to embody the standard that NTP applies when conducting a "reasonably anticipated" analysis for determining whether a chemical should be placed on the Report on Carcinogens:

> This [(e)(2)] definition of "sufficient evidence" is also well-established in the scientific community, and several references to this concept are further offered by way of illustration in the bibliography. Under these references, chemicals having sufficient evidence from animal studies have been identified as chemicals 'reasonably anticipated to be carcinogens' (NTP) . . . . When the evidence from experimental animals concerning the carcinogenicity of a chemical is not sufficient, the NTP list of carcinogens does not include it.[45]

When a chemical is nominated for the Report on Carcinogens, and thus evaluated to see if the evidence of carcinogenicity is "sufficient," the NTP makes a detailed evaluation, weighing all available information, accepting public comment, and subjecting its conclusions to peer review. First, the NTP "initially evaluates each nomination to determine whether the scientific information available for a nomination justifies its formal review and consideration." The NTP then announces which nominations are "proposed for review and solicits public comments through announcements in the Federal Register and NTP publications."[46] After receiving and responding to public and agency comments on the substances proposed for review, the NTP's formal evaluation process begins.[47] As part of that process, NTP scientists prepare additional evaluations, subject those evaluations to multiple rounds of peer

---

[45] FSR at 18-19.

[46] *Id*.

[47] *Id*.

review (both internal and external), and convene a round of public hearings.[48] Only then does the NTP reach a preliminary determination about whether a substance satisfies the "sufficient evidence of carcinogenicity" standards required for listing in the Report on Carcinogens.[49] β-myrcene was not subjected to this comprehensive NTP "sufficient evidence" review process. Importantly for β-myrcene, the Report on Carcinogens is the document in which the NTP analyzes issues such as unique, likely species specific mouse liver tumors and rat kidney tumors

If "sufficient evidence" was a conclusion expressed explicitly or inferentially by the NTP in the Technical Report, the NTP would not need to undertake its thorough review of all relevant animal studies. Instead, it simply could add chemicals to the Report on Carcinogens based on its work in the Technical Report. That is not at all what happens, however.

VI. Conclusion

β-Myrcene should not be listed as a Proposition 65 carcinogen under the authoritative bodies mechanism based solely on the results of a NTP 2-year bioassay in rats and mice. In the rat, clear evidence of carcinogenic activity in the male rat is based on kidney tumors associated with alpha 2u-globulin nephropathy. These tumors are of questionable relevance for cancer hazard identification since they represent a species- and sex-specific response that is unique to certain strains of the male rat. The clear evidence of carcinogenic activity in the male mouse is based on an increased incidence of liver tumors, common to this strain of mice and which are present even in control

[48] *Id.*

[49] *Id*.

animals at a high background rate. As in the case of the rat kidney tumors, these male mouse liver tumors are of questionable use in the cancer risk assessment for β-myrcene. Both the mouse and rat data upon which the NTP identified carcinogenic activity under the conditions of NTP's study are of dubious relevance to cancer hazard identification and this study alone does not provide sufficient proof of carcinogenic activity. OEHHA should either elect not to proceed with β-myrcene or a full review of all relevant information available for β-myrcene, including the negative genotoxicity results and protective effects, should be conducted by referring this chemical to the Carcinogen Identification Committee for analysis or special input.

Pulegone

Should Not be Listed as a Proposition 65 Carcinogen

Pursuant to the Authoritative Bodies Listing Process

Comments of

Flavor and Extract Manufacturers Association

International Chewing Gum Association

National Confectioners Association

Submitted to the
Office of Environmental Health Hazard Assessment

by

F. Jay Murray, Ph.D.
Gary M. Roberts

April 10, 2012

Table of Contents

I. Introduction 1

II. Pulegone rat data should not form the basis for cancer hazard identification 2

III. Pulegone mouse data should not form the basis for cancer hazard identification 6

A. The female mouse data should not be considered as supporting an authoritative body listing because the only response was a significant increase in benign tumors in animals dosed well in excess of the MTD 7

B. The male mouse data only provides a weak cancer signal and is not sufficient, even when viewed with other data, to support an authoritative body listing 8

C. Mouse liver tumors require additional expert analysis in cancer hazard identification because of their serious questions of relevance to humans 9

D. Recently high B6C3F1 mouse liver tumors among control animals seriously question assigning any weight to such tumors as in identification hazard 12

E. The NTP Technical Reports Review Subcommittee had differing views and interpretations of the level of carcinogenic activity in mice and rats 14

IV. Pulegone should not be listed because the NTP has not found “sufficient evidence” of carcinogenicity in animals 16

A. The NTP Technical Report Did Not Make the Required “Sufficient Evidence” Conclusion 20

V. Conclusion 22

I. Introduction

Pulegone should not be listed as a carcinogen because both the mouse and rat data upon which the NTP identified carcinogenic activity under the conditions of NTP's study were marred by excessive morbidity and mortality. The NTP did not perform an analysis that extended beyond the flawed conditions of its Technical Report data. Thus, the Flavor and Extract Manufacturers Association, the International Chewing Gum Association, and the National Confectioners Association (the "Associations") oppose listing pulegone as a Proposition 65 carcinogen.

The data suggesting carcinogenic activity for pulegone come from NTP Technical Report No. 563 (TR-563).[1] The rat data in this report should not be used as the basis for an authoritative body listing because the only dose at which tumor incidence was elevated greatly exceeded a proper Maximum Tolerated Dose (MTD). NTP itself noted that this high dose resulted in "excessive morbidity and mortality." (TR-563 at 8).[2] Similarly, the high-dose mouse data should not be used as the basis for cancer hazard identification because that dose was excessive. This results in no evidence of carcinogenicity in female mice and only limited evidence of carcinogenicity in male mice.

If California wishes to proceed with a listing evaluation of pulegone, it should do so by referring review of pulegone to the Carcinogen Identification Committee (CIC).

---

[1] National Toxicology Program (NTP). 2011. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Pulegone (CASRN 89-82-7) in F344/N Rats and B6C3F1 Mice. NTP TR 563. NIH Publication No. National Toxicology Program, Research Triangle Park, NC.

[2] *Id*. at 8.

The Associations request that the CIC review the data for pulegone before OEHHA takes any further regulatory action.

II. Pulegone rat data should not form the basis for cancer hazard identification

The NTP Technical Report found no evidence of carcinogenic activity in male rats. NTP reported clear evidence of carcinogenic activity in female rats based on "increased incidences of urinary bladder neoplasms" observed "under the conditions of these 2-year gavage studies." These Urinary bladder neoplasms, however, were observed only at doses well above the maximum tolerated dose.

One of the critical requirements of scientifically valid carcinogenicity testing in rodents is the proper selection of dose levels. However, the evidence of carcinogenicity in rats administered pulegone was observed only at an inappropriate dose level that greatly exceeded the Maximum Tolerated Dose (MTD), indicating the bladder tumors are likely secondary to the excessive mortality and morbidity observed at this dose level.

The U.S. EPA 2005 Guidelines for Carcinogen Risk Assessment (U.S. EPA Guidelines) address the issue of proper dose selection in cancer bioassays:

> "Among the many criteria for technical adequacy of animal carcinogenicity studies is the appropriateness of the dose selection."[3]
>
> "Interpretation of carcinogenicity study results is profoundly affected by study exposure conditions, especially by inappropriate dose selection."[4]

---

[3] U.S. EPA (2005) Guidelines for Carcinogen Risk Assessment. EPA/630/P-03/001F. March, 2005, p. 2-16.

[4] *Id*.

The U.S. EPA Guidelines also indicate that increases in tumors seen at excessively high doses may not be directly attributable to the test substance:

> "In addition, overt toxicity or qualitatively altered toxicokinetics due to excessively high doses may result in tumor effects that are secondary to the toxicity rather than directly attributable to the agent."[5]

The U.S. EPA Guidelines also describe an adequate high dose:

> "With regard to the appropriateness of the high dose, an adequate high dose would generally be one that produces some toxic effects without unduly affecting mortality from effects other than cancer or producing significant adverse effect on the nutrition and health of the test animals."[6]

The U.S. EPA Guidelines also identify other signs of treatment-related toxicity associated with an excessive high dose, including "significant reduction of body weight gain (e.g., greater than 10%)."[7]

And finally, the U.S. EPA Guidelines state:

> "Studies that show tumor effects only at excessive doses may be compromised and may or may not carry weight, depending on the interpretation in the context of other study results and other lines of evidence."[8]

The evidence of carcinogenicity observed in male and female rats was limited to an increase in urinary bladder tumors in female rats administered the high dose of 150 mg/kg/day of pulegone by gavage for 5 days/week. However, this level produced excessive mortality and morbidity. After 60 weeks into the 105-week study, so many of

---

[5] *Id*. at 2-16 and 2-17

[6] *Id*. at 2-17

[7] *Id.* at 2-17

[8] *Id*. at 2-18

the female rats had died at this level that the study investigators stopped exposing these animals to pulegone and the high dose rats were administered the control vehicle for the remainder of the study. Despite the early termination of exposure, none of the female rats administered 150 mg/kg/day survived to the end of the study. The early deaths were attributed to "end-stage renal disease," not cancer.[9] In addition, the body weight of the high dose females provided further evidence that the MTD had been greatly exceeded. At 60 weeks, the female rats given 150 mg/kg/day weighed 79% of the controls. The last surviving high-dose females weighed only 67% of the controls.

Given the extreme degree of mortality and morbidity observed among the high-dose female rats, the increase in urinary bladder tumors observed at this dose cannot be considered relevant to cancer hazard identification, and certainly cannot be a principal basis for the expedited authoritative bodies process. It would be highly irregular to rely upon the results of a rodent cancer bioassay for purposes of hazard identification when the only dose associated with an increase in tumors caused such severe toxicity that exposure had to be terminated about half-way through the study and no animals survived to the end of the study. Given this enormously excessive mortality and morbidity, the results of the high-dose female rats should carry no scientific weight.

It is difficult to compare the results observed in this study of female rats exposed to 150 mg/kg/day of pulegone to the results of other NTP bioassays because few, if any, NTP bioassays have ever exposed rats to such an extremely toxic dose level that so greatly exceeded the MTD. In TR-563, NTP stated: "There was clear evidence of

[9] TR-563 at 45.

carcinogenic activity of pulegone in female F344/N rats based on increased incidences of urinary bladder neoplasms." However, it is important to recognize that NTP qualified its statements regarding the level of carcinogenic activity with the phrase "under the conditions of these 2-year gavage studies." [10] NTP was obligated to summarize the results of the study even when the conditions of the study exceeded what is generally regarded to be scientifically valid testing. Importantly, NTP did not indicate whether it considered these results to be relevant for purposes of hazard identification.

The relationship between bladder tumors and kidney disease in rats has been the subject of supposition. The proximity of the bladder and the kidneys in the urinary tract raises the possibility that there is a relationship between the increased incidence of bladder tumors and the high incidence of renal disease in the high-dose female rats. NTP discussed the possibility that kidney disease from the excessive dose level of pulegone may be related to the increased incidence of bladder tumors:

> "It is currently uncertain what role the severe kidney disease (hyaline glomerulo-pathy and nephropathy) observed in the high dose animals played in the pathogenesis of the bladder tumors. In humans, chronic kidney disease has been associated with increased risk of bladder cancer (Wong *et al*., 2009). Despite the lack of a similar association in rats between kidney disease and bladder cancer, it can be hypothesized that, considering the rapid onset of hyaline glomerulopathy in the dose animals (meaning they lived a large fraction of their life with severely compromised renal function) and the unique nature of the glomerular lesion, the changes in kidney function may lead to changes in urine composition (e.g., growth factors) that are potentially related to the carcinogenic changes observed in the bladder (Cohen et al., 2007)."[11]

[10] TR-563 at 9 and 83.
[11] TR-563 at 78.

This scenario provides a plausible example of how an excessively high dose of pulegone could produce bladder tumors that are secondary to renal toxicity rather than directly attributable to pulegone.

The dramatically excessive dose (evidenced by 100% deaths for animals and severe loss of body weight at the high and mid dose levels), the absence of dose response, the absence of an effect in the males, and the presence of kidney disease should combine to remove the female rat data from consideration in Proposition 65 hazard identification.

Furthermore, additional work not available to the NTP supports the hypothesis that "the mode of action for pulegone-induced urothelial neoplasms in female rats is due to cytotoxcity and consequent regenerative cell proliferation."[12]

III. Pulegone mouse data should not form the basis for cancer hazard identification

Properly viewed, the NTP mouse data shows no scientifically valid, reliable cancer signal in females and only tumors of questionable relevance in the mid-dose of the males. Thus, the mouse data are not a sufficient basis on which to move forward with an authoritative body listing.

[12] Dodomane PR, et al., Evaluation of Urothelial Cytotoxicity of Pulegone, Poster, Society of Toxicology Annual Meeting, 2012 (attached).

A. The female mouse data should not be considered as supporting an authoritative body listing because the only response was a significant increase in benign tumors in animals dosed well in excess of the MTD

In female mice, only benign (not malignant) liver tumors were significantly increased (hepatocellular adenoma) – and only at an excessively high dose that dramatically surpassed the MTD. The high-dose level of 150 mg/kg/day clearly exceeded the MTD in female mice. The average body weight of female high-dose mice was 75% of the control mean during weeks 53-101 of the study. According to the U.S. EPA Guidelines, a decrease in body weight greater than 10% is considered to be a dose in excess of the MTD. Therefore, the interpretation of the results at the high dose in female mice is significantly affected by the inappropriate selection of an excessively high dose.

The incidence of hepatocellular carcinoma in female mice was not significantly increased compared to controls at any dose level. No statistically significant increase in the incidence of hepatoblastoma was observed at any dose level. The incidence of combined hepatocellular adenoma and carcinoma and hepatoblastoma was significantly increased at the high dose, but only because the incidence of hepatocellular adenoma was significantly increased. In other words, the statistically significant increase was driven by the incidence of benign tumors, not malignant tumors.

The “clear evidence” finding by NTP for female mice sidestepped the key consideration of the maximum dose being well over the MTD because the NTP limited its conclusion to “the conditions of these 2-year gavage studies.” Thus, the MTD information was information that NTP did not consider in its clear evidence conclusion

because the conclusion was linked to the conditions of the study. This is sufficient in itself to remove the female mouse data from relevance to the authoritative body process. The lack of a significant increase in carcinomas and the absence of any dose-response relationship further supports this conclusion.

B. The male mouse data only provides a weak cancer signal and is not sufficient, even when viewed with other data, to support an authoritative body listing

The NTP bioassay of pulegone states: "There was *clear evidence of carcinogenic activity* of pulegone in male . . . B6C3F1 mice based on increased incidences of hepatocellular neoplasms (adenomas . . . and hepatoblastomas . . .)." Hepatocellular adenomas (a benign tumor) and hepatoblastomas, and combined hepatocellular adenomas and carcinomas and hepatoblastomas were significantly increased at the middle dose (75 mg/kg/day), but not at the high dose (150 mg/kg/day). Thus, a dose-response relationship was not present for liver tumors since no statistically significant effect on any type of liver tumor or any combination of liver tumors was observed among the high dose (150 mg/kg/day) male mice. Also, the incidence of hepatocellular carcinoma was not statistically significantly increased among the male mice at any dose level. Although the body weight decreases at the high dose may have influenced the response, the absence of dose-response for the male tumors calls the weight that they should be assigned in overall hazard identification into question.

C. Mouse liver tumors require additional expert analysis in cancer hazard identification because of their serious questions of relevance to humans

Assigning weight to mouse hepatocellular tumors in a "sufficient evidence" analysis of carcinogenicity has been repeatedly challenged (Velazquez *et al*., 1996; Carmichael *et al*., 1997).[13,14] This is in part due to the fact that hepatocellular carcinoma in humans, particularly chemically-induced, is rare. In humans, the major risk factors associated with liver tumors are viral hepatitis, excessive alcohol consumption, and exposure to aflatoxin, in most cases accompanied by liver cirrhosis.

The European Food Safety Authority (EFSA) has concluded that "hepatic tumors in mice are generally considered as irrelevant for human risk assessment" in mouse dietary administration study.[15] Beginning in 2000, the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) of Australia has concluded that the liver tumors observed in B6C3F1 mice after prolonged exposure to a range of chemicals (e.g., p-dichlorobenzene) are considered to be irrelevant to humans.[16] During these evaluations, NICNAS has emphasized that the high natural spontaneous incidence of

---

[13] Velazquez SF, Schoeny R, Rice GE, Cogliano VJ (1996). Cancer risk assessment: historical perspectives, current issues, and future directions. Drug Chem Toxicol 19(3):161-185.

[14] Carmichael NG, Enzmann H, Pate I, Waechter F (1997). The significance of mouse liver tumor formation for carcinogenic risk assessment: results and conclusions from a survey of ten years of testing by the agrochemical industry. Environ Health Perspect 105(11):1196-1203.

[15] EFSA (2011). European Food Safety Authority; EFSA Statement on the scientific evaluation of two studies related to the safety of artificial sweeteners (question no EFSA-Q-2011-00064, approved on 25 February 2011 by European Food Safety Authority). EFSA J 9(2):2089 [16 pp.]. doi:10.2903/j.efsa.2011.2089. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/2089.htm.

[16] Commonwealth of Australia, 2000. National Industrial Chemical Notification and Assessment Scheme (NICNAS), December 2000, Commonwealth of Australia, 134 pp.

liver tumors in this strain and sex of mice significantly affects the ability to interpret the results.

Induction of hepatocellular tumors in mice by non-genotoxic compounds can be considered as irrelevant for cancer risk assessment.[17,18] In their evaluation of the mode of action with respect to the relevance of rodent liver tumors to cancer risk, Holsapple *et al*. (2006) concluded that in the case of chemicals displaying a phenobarbital-like P450 inducing mode of action, the observed hepatocarcinogenicity in rodents is not relevant to humans. Indeed, clinical use for over 80 years of phenobarbital, a known enzyme inducer in the rodent liver, has not been associated with an increased risk of tumor formation in the liver or any other organ in humans.[19] It is generally well accepted that male and female B6C3F1 mouse liver tumors that arise in 2-year bioassays with various agents are an indirect result of dose-related chronic toxicity and resulting cellular proliferation. In the absence of this chronic toxicity, these tumors are not considered to

[17] Holsapple, M.P., Pitot, H.C., Cohen, S.M., Boobis, A.R., Klaunig, J.E., Pastoor, T., Dellarco, V.L., Dragan, Y.P., 2006. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89, 51–56.

[18] Billington R, Lewis R.W, Mehta J.M, Dewhurst I (2010). The mouse carcinogenicity study is no longer a scientifically justifiable core data requirement for the safety assessment of pesticides Crit Rev Toxicol 40(1):35-49.

[19] McClain RM (1990). Mouse liver tumors and microsomal enzyme-inducing drugs: experimental and clinical perspectives with phenobarbital. In: Stevenson DE, Popp JA, Ward JM, McClain RM, Slaga TJ, Pitot HC, editors. Mouse Liver Carcinogenesis: Mechanisms and Species Comparisons. Symposium, Nov. 30-Dec. 3, 1988, Austin, Texas. (Progress in Clinical and Biological Research, vol 331). New York (NY): Wiley-Liss, pp. 345-365. Cited In: Carmichael et al., 1997 [Ref. #34].

represent a cancer hazard for humans, and are not adequate to establish sufficient evidence in animals.[20]

It appears that, in at least one case, NTP has called into question the relevance of mouse liver tumors for purposes of hazard identification. In an NTP bioassay (NTP TR-190), p-nitrosodiphenylamine caused "positive" findings of liver tumors in male mice and male rats:

> "Under the conditions of this bioassay, p-nitrosodiphenylamine was carcinogenic when administered in the diet to male B6C3F1 mice, causing hepatocellular carcinomas. The chemical was also carcinogenic in male Fisher 344 rats, causing liver neoplasms. No evidence was provided for the carcinogenicity of p-nitrosodiphenylamine in female B6C3F1 mice or in female Fisher 344 rats."[21]

In 1989, NTP identified p-nitrosodiphenylamine as a carcinogen in its Fifth Annual Report on Carcinogens. Subsequently, NTP delisted p-nitrosodiphenylamine for insufficient evidence of carcinogenicity in its Sixth Annual Report on Carcinogens, which was published in 1991. We are currently searching for a copy of the Sixth Annual Report on Carcinogens to further investigate the reason for delisting this substance. But, based on the results of the NTP bioassay, it is clear that the only reason for initially

---

[20] Cohen S.M., Klaunig J., Meek M.E., Hill R.N., Pastoor T., Lehman-McKeeman L., Bucher J., Longfellow D.G., Seed J., Dellarco, V. 2004. Evaluating the human relevance of chemically induced animal tumors. Toxicol. Sci. 78: 181–186.

[21] National Toxicology Program (NTP). 1979. NTP Bioassay of p-nitrosodiphenylamine for possible carcinogenicity. (NTP TR-190). National Toxicology Program, Research Triangle Park, NC.

listing it as a carcinogen was the rodent liver tumors, including the statistically significant increase in hepatocellular carcinoma in male mice. We also request that OEHHA hold open the record until we are able to obtain a copy of the Sixth Annual Report on Carcinogens and evaluate this information.

D. Recently high B6C3F1 mouse liver tumors among control animals seriously question assigning any weight to such tumors as in identification hazard

Although the NTP Technical Report states there is "clear evidence of carcinogenic activity" in male and female mice exposed to pulegone based on increased incidences of hepatocellular neoplasms, the Report did not address the issue of relevance of these mouse liver tumors to cancer hazard identification for other species or other test protocols. In fact, the high spontaneous incidence of hepatocellular tumors observed in B6C3F1 mice and the relevance of the development of these tumors in mice with regard to human cancer risk has been repeatedly questioned by scientists, including NTP scientists.[22] The background incidence of liver tumors has been steadily rising over the past decade in the B6C3F1 mice used by the NTP in its cancer bioassays. Because of their high background rate of and high degree of susceptibility to liver tumors, B6C3F1 mice are not a reliable indicator of carcinogenic hazard.

The background incidence of liver tumors in the B6C3F1 mice reported in NTP bioassays has historically been high, but in recent years, the background incidence of these tumors has significantly increased over even the historically high background rate. Prior to this recent dramatic change in the background incidence of liver tumors, the

[22] Maronpot RR, Haseman JK, Boorman GA, Eustis SL, Rao GN, Huff JE (1987). Liver lesions in B6C3F1 mice: the National Toxicology Program, experience and position. Arch Toxicol Suppl 10:10-26.

historical spontaneous incidence of liver neoplasms (combined hepatocellular adenoma and carcinomas) in control male B6C3F$_1$ mice in NTP bioassays was 32.4% with a range of 20-47%.[23] More recently, rates of combined hepatocellular adenoma and carcinomas in male B6C3F1 control mice exceeding 50% have been reported (e.g., 56% in the isoeugenol study (NTP, 2008) and 58% in the pulegone study.[24,25] Thus, the incidence of combined hepatocellular adenoma and carcinoma in the control group of male B6C3F1mice is outside the historical control range published by NTP in 2006, suggesting genetic drift in the mice used in the most recent NTP bioassays, including the bioassay of pulegone. The NTP has recognized the limitations of data pertaining to the development of liver tumors in the 2-year mouse bioassays, particularly in susceptible strains of mice (*e.g.*, B6C3F1), with respect to extrapolating the results to humans in risk assessments and has noted that alternative rodent strains are being examined to supplement rat studies.

---

[23] National Toxicology Program (NTP). 2006. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Benzophenone (CAS NO. 119-61-9) in F344/N Rats and B6C3F1 Mice. NTP TR 533. NIH Publication No. 06-4469. National Toxicology Program, Research Triangle Park, NC. http://ntp.niehs.nih.gov/

[24] National Toxicology Program (NTP). 2008. Draft Report: NTP Technical Report on the Toxicology and Carcinogenesis Studies of Isoeugenol (CAS NO. 97-54-1) in F344/N Rats and B6C3F1 Mice. NTP TR 551.

[25] National Toxicology Program (NTP). 2011. NTP Technical Report on the Toxicology and Carcinogenesis Studies of Pulegone (CASRN 89-82-7) in F344/N Rats and B6C3F1 Mice. NTP TR 563. NIH Publication No. National Toxicology Program, Research Triangle Park, NC.

E. The NTP Technical Reports Review Subcommittee had differing views and interpretations of the level of carcinogenic activity in mice and rats

The description of the levels of carcinogenic activity for both the rats and mice receiving pulegone were the subject of considerable debate. When the draft NTP Technical Report was presented to the Technical Reports Review Subcommittee, the NTP staff proposed the following conclusion: "no evidence of carcinogenic activity of pulegone in male F344/N rats, some evidence of carcinogenic activity of pulegone in female F344/N rats, and clear evidence of carcinogenic activity of pulegone in male and female B6C3F1 mice." However, when the Technical Reports Review Committee voted on a motion to accept this characterization of the level of carcinogenic activity, the motion failed to carry.[26] Two revisions were proposed by the Technical Reports Review Committee. First, a proposal was made to change the statement on mice to "clear evidence based on increased incidences of hepatocellular neoplasms (adenomas in both sexes and hepatoblastomas in males)." Second, it was proposed that the statement for female rats be changed from "clear evidence of carcinogenic activity" to "some evidence of carcinogenic activity."[27]

When the proposed revisions to the final statement were brought to a vote, there was widespread disagreement among the members of the Technical Reports Review Panel. The revisions passed by a narrow margin of 6 to 4 votes. Several members voted against the motion because they did not believe there was "clear evidence of carcinogenic activity" in female rats. At least one member expressed concern that the

---

[26] TR-563 at 15-16.

[27] *Id.*

increase in bladder tumors in female rats occurred at a dose that exceeded the MTD. Two members disagreed with the statement regarding mouse liver tumors.

Importantly, members of the Technical Reports Review Panel appeared to express concern that the Technical Report include a statement that makes it clear that the Technical Report was not designed to consider the implications of the results in mice and rats for humans. For example, one reviewer (Dr. Auerbach) replied that the dose selection for this study included consideration of a possible adaptive response to glutathione depletion, and the hyaline glomerulopathy was not fully diagnosed until a retrospective analysis of the short-term studies was conducted after the 2-year studies were completed. It appears that this reviewer was making the point that new data may indicate that these findings are not relevant to humans. Dr. J.R. Bucher (NIEHS) responded to the reviewer's comment by noting that the Foreword to the report indicates that risk assessment is beyond the purview of these studies. A second reviewer (Dr. Teeguarden) also suggested that language be included in the report clarifying that NTP Technical Reports are not risk assessment documents.

Although we are relying on a summary of the meeting of the Technical Reports Review Panel, it appears members of the Technical Reports Review Panel were concerned that the results in rodents administered pulegone may not be relevant for cancer hazard identification. Such a determination is one of the early steps in risk assessments conducted by NTP in its Report on Carcinogens. We have requested a copy of the full transcript of the Technical Reports Review Panel discussion to determine whether there was additional, relevant discussion on this topic. We request that the record for pulegone be held open until we obtain this transcript from NTP.

IV. Pulegone should not be listed because the NTP has not found "sufficient evidence" of carcinogenicity in animals

OEHHA does not have the authority to list pulegone as a carcinogen because the NTP did not "conclude" that pulegone "causes cancer" in animals.[28] The "primary" Proposition 65 listing mechanism for candidate carcinogens is review by the "state's qualified experts," the Carcinogen Identification Committee (CIC).[29] The "authoritative body" listing mechanism is supposed to be a shortcut, allowing listing without CIC review where an authoritative body has already done the work that the CIC would otherwise be required to do.[30] As relevant here, that mechanism is triggered only when a chemical has been "formally identified by an authoritative body as causing cancer" in a report which "concludes" that "[s]ufficient evidence of carcinogenicity exists from studies in experimental animals."[31] To constitute a "sufficient evidence" finding, the authoritative body's formal "report" must "conclude[]" that "studies in experimental animals indicate that there is an increased incidence of [cancer]."[32] OEHHA is not authorized to substantively evaluate the data on pulegone and conclude on its own that "sufficient evidence" of carcinogenicity exists. OEHHA's role is limited by regulation to the "ministerial" task of reviewing the authoritative body's formal reports and

---

[28] 27 CCR § 25306(a), (d)(1), and (e)(2).

[29] See Final Statement of Reasons ("FSR") for 27 CCR § 25306 (then 22 CCR § 12306) at 8.

[30] *Id.* at 5, 8.

[31] 27 CCR § 25306(a), (d)(1), (e)(2).

[32] 27 CCR § 25306(e)(2).

determining whether the authoritative body has, itself, issued a qualifying sufficient evidence "conclu[sion]."[33]

NTP has never "conclude[d]" that "sufficient evidence of carcinogenicity exists from studies in experimental animals" within the meaning of section 25306 for pulegone. Rather, the NTP expressed four separate and limited conclusions about carcinogenic activity in one strain of mice and one strain of rats *under the conditions of its experiment*. Moreover, NTP said that the rats it studied experienced "excessive morbidity and mortality,"[34] which further emphasizes the limited nature of the NTP statements and the absence of a "sufficient evidence" finding. NTP stated that "[t]he interpretative conclusions presented in NTP Technical Reports are based only on the results of these NTP studies. Extrapolation of these results to other species requires analyses beyond the intent of these reports."[35] NTP does evaluate chemicals for "sufficient evidence" of carcinogenicity in studies of experimental animals, applying a standard equivalent to section 25306(e)(2), but its current practice is to do so when evaluating chemicals for inclusion in its "Report on Carcinogens."

The plain language of section 25306 equates "sufficient evidence" with what "studies in experimental animals indicate" generally, and the regulatory history makes clear that this standard was intended to mirror the scientific consensus on sufficient evidence reflected in the language California borrowed directly from the EPA's 1986

---

[33] FSR at 8.

[34] *Id*.at 8.

[35] *Id*. at Foreword.

Guidelines for Carcinogen Risk Assessment.[36] Those Guidelines require consideration of all relevant studies, not just individual studies in isolation. OEHHA's interpretation would require it to list a chemical on the basis of a single positive study—even if other Technical Reports summarize equally valid, or more valid, data that calls into question the single positive study. NTP almost certainly would not agree in those circumstances that "studies in experimental animals indicate that there is an increased incidence of [cancer]."[37]

To the extent the language leaves any doubt, the regulatory history dispels it. It is undisputed that section 25306(e)'s "causing cancer" definition regarding animal evidence is the well known "sufficient evidence" test taken from the EPA's 1986 Guidelines for Carcinogen Risk Assessment, with the "same or substantially similar criteria" in use by the NTP, the authoritative body in question.[38] The FSR explains the regulation, repeatedly emphasizing that "sufficient evidence" is not a new standard for OEHHA scientists to administer or for industry scientists and observers to understand, but instead a standard already used by authoritative bodies to make their own cancer causing determinations. The FSR states:

> Subsection (e) provides that, for purposes of section 12306 [now 25306], the phrase "as causing cancer" means that either of two scientific criteria have been satisfied. Generally, the authoritative body may *rely* on either studies in humans or studies in animals. These criteria are consistent with the criteria the Panel presently uses in evaluating chemicals for listing. The Panel utilizes the EPA's

[36] 27 CCR § 25306(e)(2); FSR at 15 (language drawn from EPA Guidelines)

[37] 27CCR §§25306(e)(2).

[38] *Compare* 27 CCR § 25306(e)(2) *with* 1986 EPA Cancer Guidelines at 33999.

> Classification System for Categorizing Weight of Evidence for Carcinogens From Humans and Animal Studies (51 Fed. Reg. 33999 (Sept. 24, 1986)). The same, or substantially similar criteria have been adopted by many regulatory agencies and scientific organizations involved in hazard identification. The use of these criteria will ensure that *the standards applied by an authoritative body* are the same as or substantially similar to those used by the Panel to evaluate chemicals.[39]
>
> * * * *
>
> It is not the intention of the Agency to *substitute its scientific judgment for that of the authoritative body*. The Agency's inquiry will be limited to whether the authoritative body *relied upon* scientific data in an amount sufficient *to conclude that the chemical causes cancer*. . . . *Because the body is considered authoritative, and the body utilizes the same or substantially the same criteria as set forth in section (e)*, it will be assumed that the data relied upon is scientifically valid. The Agency will look to determine whether the authoritative body relied upon animal or human data in an amount sufficient to satisfy the criteria. If so, the chemical will be proposed for listing.[40]

These FSR passages make it clear that the California Health and Welfare Agency, which wrote the regulation, expected the sufficient evidence standard would be "applied" by the authoritative body to "conclude that the chemical causes cancer." These two passages emphasize that the authoritative body is expected to exercise judgment in making the ultimate "causing cancer" conclusion according to substantially the same criteria as set forth in paragraph (e).

The 1986 EPA Guidelines for Carcinogen Risk Assessment provide that a "sufficient evidence" determination cannot be based on the results of individual animal

---

[39] FSR at 15 (emphasis added).

[40] FSR at 15, 18 (emphasis added).

studies considered in isolation, but must be based on a broader review of relevant data. EPA summarizes its standard as follows: "At various points in the above discussion, EPA has emphasized the need for an overall, balanced judgment of the totality of the available evidence."[41] The EPA Guidelines also state that "[r]eplicate negative studies that are essentially identical in all other respects to a positive study may indicate that the positive results are spurious."[42] [43]

Thus, the EPA cancer risk assessment guidelines, from which section (e)(2) was taken, require that all relevant "studies" be considered as a whole in making a "sufficient evidence" determination, whether based on animal or human data. Section (e)(2) was intended to implement the same standard. The regulation's copied language and the FSR make this abundantly clear. The NTP has not yet performed that overall analysis for pulegone, and thus its Technical Report does not contain a "sufficient evidence" determination required to support an authoritative body listing, or to render the CIC's consideration of pulegone unnecessary.

A. The NTP Technical Report Did Not Make the Required "Sufficient Evidence" Conclusion

The record demonstrates that the NTP did not make a "sufficient evidence" finding with regard to pulegone. The Technical Report expresses carcinogenicity

---

[41] 51 Fed. Reg. 33992, 33996 (Sept. 24, 1986).

[42] *Id.* at 33995 (middle column).

[43] The EPA Guidelines also state expressly that the classification scheme "is not meant to be applied rigidly or mechanically," whenever there questionable positive data, but instead provides that "Results and conclusions concerning the agent, derived from different types of information, whether indicating positive or negative responses, are melded together…." *Id*. at 33996 (left column), 33994 (left column).

conclusions limited to "the conditions of these 2-year feed studies."[44] It does not render an overall conclusion about what "studies in experimental animals indicate." The Technical Report warns that its conclusions are not to be extrapolated "to other species, including characterization of hazards and risks to humans" because doing so would require "analyses beyond the intent" of the report. (*Id*.)

The Final Statement of Reasons expressly confirms, twice, that the "sufficient evidence" standard of section 25306(e) is meant to embody the standard that NTP applies when conducting a "reasonably anticipated" analysis for determining whether a chemical should be placed on the Report on Carcinogens:

> This [(e)(2)] definition of "sufficient evidence" is also well-established in the scientific community, and several references to this concept are further offered by way of illustration in the bibliography. Under these references, chemicals having sufficient evidence from animal studies have been identified as chemicals 'reasonably anticipated to be carcinogens' (NTP) . . . . When the evidence from experimental animals concerning the carcinogenicity of a chemical is not sufficient, the NTP list of carcinogens does not include it.[45]

When a chemical is nominated for the Report on Carcinogens, and thus evaluated to see if the evidence of carcinogenicity is "sufficient," the NTP makes a detailed evaluation, weighing all available information, accepting public comment, and subjecting its conclusions to peer review. First, the NTP "initially evaluates each nomination to determine whether the scientific information available for a nomination justifies its formal review and consideration." The NTP then announces which

[44] TR-563 at 9 and 83.
[45] FSR at 18-19.

nominations are "proposed for review and solicits public comments through announcements in the Federal Register and NTP publications."[46] After receiving and responding to public and agency comments on the substances proposed for review, the NTP's formal evaluation process begins. As part of that process, NTP scientists prepare additional evaluations, subject those evaluations to multiple rounds of peer review (both internal and external), and convene a round of public hearings. Only then does the NTP reach a preliminary determination about whether a substance satisfies the "sufficient evidence of carcinogenicity" standards required for listing in the Report on Carcinogens. Pulegone was not subjected to this comprehensive NTP "sufficient evidence" review process.

If "sufficient evidence" was a conclusion expressed explicitly or inferentially by the NTP in the Technical Report, the NTP would not need to undertake its thorough review of all relevant animal studies. Instead, it simply could add chemicals to the Report on Carcinogens based on its work in the Technical Report. That is not at all what happens, however.

V. Conclusion

When examined closely, there is not "sufficient evidence" of carcinogenicity on pulegone to list this substance through the authoritative bodies process. The proposed basis for listing, the NTP Report, is inadequate because the MTD was greatly exceeded and the NTP Report's conclusions are tied to this inappropriate dosing. Similarly, the growing consensus that mouse liver tumors should not be an important or primary basis

[46] *Id*.

for cancer hazard identification also should result in this administrative listing not moving forward. The Associations request the opportunity to meet the with OEHHA and discuss this matter further after OEHHA has had an opportunity to review the information presented above.

30389817\V-3

Evaluation of the Urothelial Cytotoxicity of Pulegone

Dodmane PR, Da Rocha MS, Arnold LL, Pennington KL, Anwar MM, Adams BR#, Taylor SV*, Adams TB*, Cohen SM
Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA
*Flavor and Extract Manufacturers Association, Washington, DC, USA
#Departments of Radiation Oncology and Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA, USA

UNIVERSITY OF NEBRASKA MEDICAL CENTER

ABSTRACT

Essential oils, from mint plants including peppermint and pennyroyal oils, are used at low levels (<20 ppm) as flavoring agents in various foods and beverages. At high levels (>5 g) pennyroyal oil poisoning can cause adverse health effects including death. Pulegone, a monoterpene ketone, is a major component of these oils. A major metabolite menthofuran, is implicated in its toxicity. In a 2-year bioassay, oral administration of pulegone slightly increased the urothelial tumor incidence in female rats. We hypothesized that pulegone causes urothelial cytotoxicity and increases urothelial cell proliferation, ultimately leading to tumors. We administered pulegone by oral gavage at 0, 75 or 150 mg/kg body weight to female rats for 4 weeks. Fresh void urine was analyzed for the presence of abnormal crystals. Urinary bladders were evaluated by light and scanning electron microscopy (SEM), and the bromodeoxyuridine (BrdU) labeling index. *In vitro*, pulegone and its metabolites, menthofuran and menthone, were tested for cytotoxicity in MYP3 rat urothelial cells by the MTT assay. Rats in the pulegone treated groups had urogenital staining and alopecia, and alopecia around the mouth. No abnormal urinary crystals were found by light microscopy. By SEM, bladders from the 75 and 150 mg/kg treated rats showed necrosis and exfoliation in 2/9 and 4/10 bladders, respectively. There was a significant increase in the BrdU labeling index in the high dose group. *In vitro*, pulegone, menthofuran and menthone, had LC50s of 0.27 mM, 1.42 mM and 4.50 mM, respectively. In conclusion, pulegone administration resulted in necrosis, exfoliation and increased cell proliferation in the rat bladder urothelium. *In vitro*, pulegone was more toxic to rat urothelial cells compared to its metabolites. These results suggest that pulegone induced urothelial effects may be due to pulegone and its metabolites in the urine.

BACKGROUND

- Present in essential oils from mint plants including peppermint and pennyroyal, which are used at low levels as flavoring agents in foods and beverages (1).
- Pennyroyal oil poisoning can cause adverse health effects, including death (2, 3).
- (R)-(+)-Pulegone, a monoterpene ketone, is a major constituent (60-90%) of pennyoryal oil, present in high amount (~4%) in peppermint oil and (~2%) in mint oil (1).
- In a 2-year bioassay, pulegone (p.o) slightly increased urothelial tumor incidence in female rats (1).
- Pulegone metabolites are excreted in urine (4,5).
- We hypothesized that pulegone causes urothelial cytotoxicity and increased cell proliferation, ultimately leading to tumor formation.

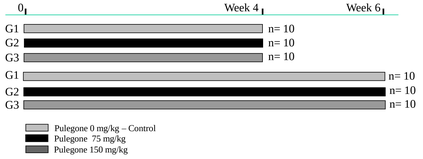
OBJECTIVE

- Investigate the effect of oral administration of pulegone on urine and urothelium and determine the urinary concentration of pulegone and its metabolites.
- Investigate the cytotoxicity of pulegone and its metabolites on rat urothelial (MYP3) and human urothelial (1T1) cells, and compare to urinary concentrations.

MATERIALS AND METHODS

TEST MATERIALS
(R)-(+)-pulegone, (+)-menthofuran and menthone were purchased from Sigma-Aldrich (St. Louis, MO). Piperitone was donated by Givaudan Schweiz, AG (Dubendorf, Switzerland) and piperitenone was a gift from Nippon Terpene Chemicals (Tokyo, Japan). All the chemicals were stored at 4 to 8°C in dark.

IN VIVO
Test Animals
6-week old female F344/N rats (Charles River Breeding Laboratories, Portage, MI)
Basal Diet
Powdered irradiated NTP-200 rodent diet (Zeigler Bros, Gardnes, PA)
Treatment
Pulegone was administered by oral gavage 5 days/week at concentration of 75 or 150 mg/kg body weight in corn oil.

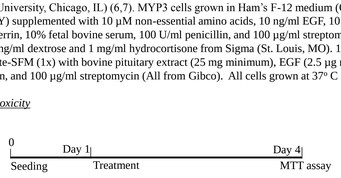


Parameters evaluated
* Freshly voided urine
  - Urinary pH (Microelectrode, Microelectrodes Inc., Bedford, NJ)
  - Evaluation of urinary sediment by light microscopy
* 18-hour urines
  - Urine volume, creatinine (Beckman Coulter DxC 800, Beckman Coulter Inc., Brea, CA)
  - Pulegone and its metabolites (LC-MS/MS, International Flavors & Fragrances Inc., Union Beach, NJ)
• Bladder urothelium
  - Light microscopy
    - A diagnosis of simple hyperplasia was made when there were 4-5 cell layers in the urothelium.
  - SEM
    - Class 1 bladders-flat, polygonal superficial urothelial cells; class 2 bladders- occasional small foci of superficial urothelial necrosis; class 3 bladders-numerous small foci of superficial urothelial necrosis; class 4 bladders-extensive superficial urothelial necrosis, especially in the dome of the bladder; class 5 bladders-necrosis and piling up (hyperplasia) of rounded urothelial cells.
  - Proliferative activity of urothelium
    - Bromodeoxyuridine (BrdU) immunohistochemistry – BrdU injected i.p. 1 h before necropsy. Anti-BrdU (Millipore Corp., Temecula, CA) diluted 1:2000. The number of BrdU-labeled cells in at least 3000 urothelial cells was counted.

IN VITRO
Cell culture
Cytotoxicity evaluated in MYP3 rat urothelial cell line and 1T1 human urothelial cell line (Dr. Ryoichi Oyasu, Northwestern University, Chicago, IL) (6,7). MYP3 cells grown in Ham's F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10 µM non-essential amino acids, 10 ng/ml EGF, 10 µg/ml insulin, 5 µg/ml transferrin, 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco) and 250 mg/ml dextrose and 1 mg/ml hydrocortisone from Sigma (St. Louis, MO). 1T1 cells cultured in Keratinocyte-SFM (1x) with bovine pituitary extract (25 mg minimum), EGF (2.5 µg minimum) and 100 U/ml penicillin, and 100 µg/ml streptomycin (All from Gibco). All cells grown at 37° C in 5% CO_2.

Determination of cytotoxicity

Experimental Design:



MYP3 seeding concentration - 4000 cells/well in a 96-well plate.
1T1 seeding concentration - 6000 cells/well in a 96-well plate.
Treatment started 24 hrs after seeding and continued for 3 days without changing medium.
Cell viability determined by MTT assay. Percent survivability calculated as the ratio of the mean cell number in 4 treated wells to that in the 4 control wells. Data graphed with the known concentrations of the test material on x-axis and percent survivability at those concentrations on y-axis. The LC_{50} calculated by non-linear regression analysis of the data using GrapPad Prism version 5.0 for windows (GraphPad Software, San Diego, CA)

RESULTS

In Vivo
General findings:
1. No treatment-related mortality
2. Animals showed alopecia around the mouth and yellow staining and alopecia around urogenital area
3. Mean body weights of rats treated with 150 mg/kg pulegone were slightly lower than 75 mg/kg pulegone and control groups from Day 6 onward
4. No difference in food consumption between groups
5. Water consumption was significantly increased in 75 and 150 mg/kg pulegone-treated groups compared to control

Urinary changes :
1. Urine collected on Day 19 had lower mean urinary pH in pulegone-treated groups.
2. No crystals found in urines from any group other than a few struvite crystals (normal occurrence in rat)
3. 18-h urine, collected during week 6, had significantly increased volume in 150 mg/kg treated group compared to control. Creatinine levels in the same group showed significant decrease
4. Gas chromatographic analysis revealed presence of pulegone, piperitone, piperitenone and menthofuran in both treated groups (see Table 2)

Histopathology:
1. After 4 weeks of treatment, there was no significant changes in the bladder epithelium by light microscopy
2. No changes were found in kidneys, mild to moderate single-cell necrosis was observed in all livers of 150 mg/kg group

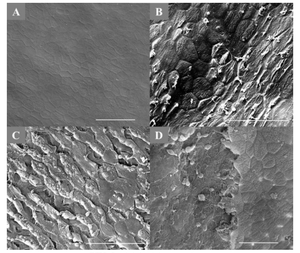
Immunohistochemical determination of BrdU:
1. BrdU labeling index in urothelium of 150 mg/kg group was significantly increased compared to control. Though the index was increased in 75 mg/kg group, it was not statistically significant

SEM examination of the bladder epithelium:
1. There was a gradual dose related increase in the classification of the bladder epithelium
2. Necrosis and exfoliation with 75 mg/kg pulegone treatment
3. Extensive necrosis and exfoliation 150 mg/kg pulegone treatment
4. Severity of lesions increased with increase in dose

Table 1.

Treatment	Histopathology: Normal	Histopathology: Simple Hyperplasia	BrdU Labeling Index (%) Mean ± S.E. (n)	SEM Classification 1	2	3	4	5
0 mg/kg Pulegone	10	0	0.03±0.02 (10)	7	2	1	0	0
75 mg/kg Pulegone	9	1	0.09±0.05 (7)	3	1	3	2	0
150 mg/kg Pulegone[a]	10	0	1.13±0.18 (8)[b]	0	3	3	4	0

[a] SEM classification significantly different compared to control group, $p<0.05$
[b] Significantly different compared to control group, $p<0.05$



SEM of bladder surface

A: Pulegone 0 mg/kg – Control (400X, 100 µm)
B: Pulegone 75 mg/kg (629X, 100 µm)
C: Pulegone 150 mg/kg (600X, 100 µm)
D: Pulegone 150 mg/kg (800X, 50 µm)

RESULTS

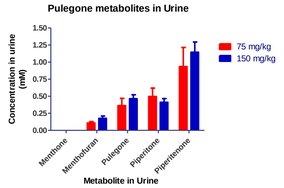
Urinary metabolite analysis:
- Piperitenone was the major metabolite detected in urine
- Unmetabolized pulegone was present at high concentration in the urine
- Concentrations of pulegone, piperitenone and piperitone in urine were higher than LC50 values, suggesting they may be involved in the urothelial cytotoxicity observed in vivo

In vitro cytotoxicity
- Pulegone was more cytotoxic compared to its metabolites on MYP3 cells
- Piperitenone was more toxic than pulegone and other metabolites in 1T1 cells

Table 2.

Metabolite	*In vivo* urinary concentration (mM): 75 mg/kg	150 mg/kg	*In vitro* cytotoxicity (LC50 in mM): MYP3 cells	1T1 cells
Pulegone	0.36±0.11	0.46±0.06	0.27	0.57
Piperitenone	0.93±0.28	1.15±0.15	0.50	0.44
Piperitone	0.50±0.12	0.41±0.05	1.16	1.29
Menthofuran	0.11±0.02	0.18±0.03	1.41	3.60
Menthone	ND[a]	ND[a]	4.50	7.25

[a] Not detected



CONCLUSION

These data support the hypothesis that the mode of action for pulegone-induced urothelial neoplasms in female rats is due to cytotoxicity and consequent regenerative cell proliferation. Pulegone and its major metabolites are concentrated and excreted in the urine in rats administered high doses, present in urine at cytotoxic concentration.

REFERENCES

1. National Toxicology Program. 2011. Toxicology and carcinogenesis studies of pulegone in F344/N rats and B6C3F1 mice (gavage studies). Natl. Toxicol. Program Tech. Rep. Ser., 563:1-201.
2. Opinion of the Scientific Committee on food on pulegone and menthofuran, 2002, Brussel, Belgium.
3. Anderson, I., Mullen, W. and Meeker, J. 1993. Pennyroyal toxicity: measurement of toxic metabolite levels in two cases and review of the literature. Ann. Intern. Med., 124:726-734.
4. Chen, L., Lebetkin, E. and Burka, L. 2001. Metabolism of (R)-(+)-pulegone in F344 rats. Drug Metabolism and Disposition, 29(12):1566-77.
5. Madyastha, K. and Gaikwad, N. 1998. Metabolism of S-(-)-pulegone in rat. Xenobiotica, 28(8):723-34.
6. Kawamata, H., Kameyama, S., Nan, L., Kawai, K., Oyasu, R., 1993. Effect of epidermal growth factor and transforming growth factor beta 1 on growth and invasive potentials of newly established rat bladder carcinoma cell lines. Int. J. Cancer 55, 968-973.
7. Tamatani, T., Hattori, K., Nakashiro, K., Hayashi, Y., Wu, S., Klumpp, D., Reddy, J. K., Oyasu, R., 1999. Neoplastic conversion of human urothelial cells in vitro by overexpression of H2O2-generating peroxisomal fatty acyl CoA oxidase. Int. J. Oncol. 15, 743-749.

UNIVERSITY OF Nebraska Medical Center

Isopyrazam

Response to the OEHHA Request for Relevant Information on Isopyrazam As a Chemical Being Considered for Listing on Proposition 65 by the Authoritative Bodies Mechanism

DATA REQUIREMENT(S): Not Applicable

AUTHOR(S): Rich Peffer, Ph.D,
Debra S. Stubbs

COMPLETION DATE: April 10, 2012

SPONSOR(S): Syngenta Crop Protection, LLC
410 Swing Road
Greensboro, NC 27409

1.0 INTRODUCTION

The California Office of Environmental Health Hazard Assessment (OEHHA) requested relevant information regarding whether isopyrazam meets the criteria for listing as known to the state to cause cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986 also known as Proposition 65. This action is being proposed under the authoritative bodies listing mechanism process under Proposition 65.

Isopyrazam is a pyrazole carboxamide and belongs to the ortho-substituted phenyl amides (OPAs) class of fungicides. The active ingredient was submitted to the USEPA to establish an import tolerance on banana treated in Central and South America. It is used to control black sigatoka in banana which is a disease that causes leaf spotting that can ultimately lead to significantly reduced yields and / or plant loss for the grower.

The purpose of this document is to provide information regarding the potential listing.

2.0 CRITERIA TO IDENTIFY ISOPYRAZAM FOR LISTING UNDER THE AUTHORITATIVE BODIES MECHANISM OF PROPOSITION 65

According to the February 10, 2012 notice OEHHA indicates USEPA published a report on ispoyrazam, entitled *Cancer Assessment Document, Evaluation of the Carcinogenic Potential of Isopyrazam* during the evaluation of isopyrazam for an import tolerance petition for use on bananas. USEPA, which is identified as an authoritative body, concluded that isopyrazam is "likely to be carcinogenic to humans". The notice indicates that this report appears to meet the requirements for a formal identification and sufficiency of evidence criteria in the Proposition 65 regulations. The USEPA classification is based on the presence of thyroid follicular cell tumors in male rats, and liver and uterine tumors in female rats at doses adequate to evaluate the carcinogenic potential of isopyrazam.

3.0 RELEVANT INFORMATION FOR CONSIDERATION OF SUFFICIENCY FOR LISTING

Syngenta is currently conducting studies to understand the mode of action for high-dose tumors in rats with isopyrazam. These mechanistic studies are designed to identify the key events that occur in rats at earlier time points and are necessary steps in the process leading to tumor formation. An understanding of the mode of action can be used to determine a threshold dose below which tumors will not occur, and aid in determining whether the rodent mode of action is relevant to humans. If it is determined that the mode of action is not relevant to humans then it is anticipated that EPA would re-evaluate the current toxicity profile and change their current cancer classification.

4.0 CONCLUSIONS

The notice indicated that isopyrazam is not registered by USEPA for use as a fungicide in the United States and therefore, it cannot be sold nor distributed for sale. It is registered for use in South and Central America to control black sigatoka, a potentially devastating disease on bananas. There is no direct exposure to the residents of California because there is no product that contains this chemical available in the state. The only possible exposure to residents of California is indirectly through potential residues on imported bananas. However, potential residues have been demonstrated to be low since USEPA established import tolerances for isopyrazam in banana of only 0.05 mg/kg. In addition USEPA has evaluated the potential residues on imported bananas and concluded in the Final Rule published in the Federal Register on October 5, 2011, that "there is a reasonable certainty that no harm will result to the general population or to infants and children from aggregate exposure to isopyrazam residues." Syngenta believes it is not necessary to list isopyrazam on Proposition 65, because there is no potential for direct exposure as noted above.