FLAVOR AND EXTRACT MANUFACTURERS
ASSOCIATION OF THE UNITED STATES
Suite 925, 1620 I Street, NW, Washington, DC 20006
Phone: (202) 293-5800 Fax: (202) 463-8998
www.femaflavor.org

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Dr. Ruth Lunn
Director, ORoC, DNTP
National Institute of Environmental Health Science
P.O. Box 12233
MD K2-14
Research Triangle Park, NC 27709

Nominations to the Report on Carcinogens; Request for Information
78 Fed. Reg. 22890 20 September 2013

Dear Dr. Lunn:

The Flavor and Extract Manufacturers Association of the United States (FEMA) is pleased to provide information relevant to the proposed listing of pulegone in a future edition of the Report on Carcinogens (RoC).

The Flavor and Extract Manufacturers Association of the United States

FEMA, founded in 1909, is the Washington, D.C.-based national association of the U.S. flavor industry. FEMA's members include flavor manufacturers, flavor users, flavor ingredient suppliers, and others with an interest in the U.S. flavor industry. FEMA's flavor manufacturing members include all of the largest flavor manufacturers and FEMA's flavor manufacturing members produce >95% of all flavors consumed in the U.S. FEMA and its members are committed to assisting flavor manufacturers in providing an ample supply of safe and wholesome flavors for inclusion in foods, beverages, pharmaceuticals, and other consumer products.

Relevant Information

Pulegone is among the compounds under consideration by the National Toxicology Program (NTP) for listing in a future edition of the Report on Carcinogens. The Report on Carcinogens is a "congressionally mandated, science-based public health report that identifies agents, substances, mixtures or exposures in our environment that pose a cancer hazard for people in the United States". As explained

below, pulegone does not present a cancer hazard for people in the United States and should not be listed in a future edition of the RoC.

The basis for the inclusion of pulegone in this list of chemicals stems from reported hepatic tumors in male and female mice and urinary bladder tumors in female rats, in 2-year bioassays conducted by the NTP (2011). In the 2-year rat study, there were no treatment-related increases in the incidences of neoplasms in males; small but significant increases in the incidences of urinary bladder papilloma and of papilloma or carcinoma (combined) were seen only in females and only at the top dose (150 mg/kg stop-exposure dose). Upon reviewing the overall evidence of both studies, the NTP concluded that there was "clear evidence" of carcinogenic activity of pulegone in male and female B6C3F1 mice based on increased incidences of hepatocellular neoplasms and "clear evidence" of carcinogenic activity of pulegone in female F344/N rats based on increased incidences of urinary bladder neoplasms (NTP 2011).

There are several reasons against concluding that pulegone presents a carcinogenic risk to humans, including evidence from genotoxicity studies, metabolic fate, and overall evidence of its toxicity profile in rats and mice. The Maximum Tolerated Dose (MTD) was clearly exceeded in the rodent bioassays indicating tumor development secondary to significant toxicity. In fact, considerable disagreement among experts of the NTP's Technical Report Review Panel on the validity of the two rodent bioassays in extrapolating human cancer risk from pulegone intake has been documented in the finalization of the technical report. A summary of the reasons why the evidence of carcinogenicity is not sufficient and the observations in rodents are not relevant to human cancer risk are presented below.

A- Pulegone (as well as its proximate cytotoxic metabolite menthofuran) has tested negative for mutagenicity in three separate bacterial Ames assays *in vitro* in *Salmonella typhimurium* strains TA 97, TA98, TA100, and TA1535, and in *Escherichia coli strain* WP2 *urvA*/pKM101, all in the presence and absence of metabolic activation (S9) (Andersen and Jensen, 1984; NTP, 2011). A positive result was reported with activation in a third experiment in *S. typhimurium* strains TA98 (very weak) and in *E. strain* WP2 *urvA*/pKM101 (NTP, 2011), but its validity and significance is questionable for the following reasons: a) only one strain of *S. typhimurium* was selected, b) the concentrations at which higher number of revertants were reported were within the range of the previous two assays in which the material was clearly negative, c) the discrepancy in the results of the third assay compared to the previous two assays was not explained by protocol differences or any other speculation in the final NTP report. Furthermore, pulegone was tested negative for genotoxicity in the *in vivo* micronucleus assay in mice up to 150 mg/kg bw (NTP, 2011). The evidence supporting absence of genotoxicity outweighs the evidence supporting genotoxicity.

B- At high dose levels (250-400 mg/kg bw), pulegone is metabolized to menthofuran (Moorthy *et al*., 1989a, b; Thomassen *et al*., 1988, 1990, 1991; Madyastha and Raj, 1990, 1991, 1992, 1993). Also at high dose levels, menthofuran, in turn, yields a proximate reactive hepatotoxic metabolite {2-Z-(2'-keto-4'-methylcyclohexyidene)propanal} which has been shown to react directly with liver proteins (Chen et al., 2003b). This is consistent with all prior evidence that showed that it is the reactive menthofuran metabolites that bind to liver proteins and are believed to be responsible for the hepatotoxicity of pulegone and menthofuran seen at high dose levels (McClanahan *et al*., 1989; Thomassen *et al*., 1992). At lower dose levels, primary

hydroxylated pulegone metabolites are efficiently eliminated following glutathione and glucuronic acid conjugation, minimizing further transformation to menthofuran and subsequent reactive metabolites (Chen et al., 2003b). Glutathione-depleting agents have been shown to increase pulegone hepatotoxicity in mice (Chen et al., 2003b), therefore indicating that reactive metabolites increase with increasing dose above a threshold where glutathione conjugation is overcome in rodents.

C- Short-term toxicity studies have revealed adverse effects that are evident biological precursors of the pathologies observed to develop in the chronic toxicity studies. Hepatotoxicity, in the form of increased absolute and relative liver weights, hepatocyte hypertrophy and bile duct hyperplasia, was reported only at high dose levels (150 mg/kg/day) in B6C3F1 mice and F344N Fischer rats (and in 75 mg/kg/day male rats) treated for 90 days (NTP, 2011). A progressive development of hepatotoxicity is indicated by the dose-dependent increase in glutathione levels and other liver parameters and is consistent with an adaptive metabolic response to increasing pulegone dose and its associated metabolic products. Increases in other organ weights, observations of hyperplasia and soft tissue mineralization also indicate dose-dependent toxicity with exceptional sensitivity of the rat kidney, due to sex- and species-specific responses (susceptibility to chronic nephropathy and processing of α-2u-globulin aggregates). These effects demonstrated a clear threshold of toxicity with a no-observable-adverse effect level (NOAEL) of 9.375 mg/kg bw/day in rats, on the basis of relative liver weight increase in males and a NOAEL of 37.5 mg/kg/day in mice, on the basis of liver weight and glutathione level increase (NTP, 2011).

D- Tumors reported in the rat and in the mouse occurred only at very high dose levels that clearly exceeded the definition of Maximum Tolerated Dose (MTD). Specifically, in rats severe morbidity and mortality caused the cessation of treatment half way through the study, at 60 weeks of a 105 week total study time. In addition, in both rats and mice, reduction of body weight exceeded the acceptable 10% limit that indicates severe toxicity (U.S. EPA, 2005). Therefore, even though tumors were observed under the conditions of these studies, the conditions are such that resulted in excessive toxicity. NTP's conclusion is limited to these conditions without specification of their validity in extrapolating cancer risk to humans.

E- Given the absence of genotoxicity (item A above), the evidence of metabolic transformation to reactive metabolites above a dose threshold, the mechanism of tumor development in both mice and rats secondary to cytotoxicity, as demonstrated by the histopathological examination of the lesions and the association of tumor development with doses exceeding the MTD, the evidence of pulegone's carcinogenic potential is not relevant to low levels of consumer intake. All toxicity studies, including the 2-year NTP bioassays are consistent with tumor development above a certain threshold of toxicity (~9 mg/kg bw/day).

F- Furthermore, a closer examination of the profile of tumors reported in mice and rats reveals that there are several reasons to conclude that neither the hepatic tumors in mice nor the urinary bladder neoplasia in female rats are relevant to human cancer risk. Considerations for each 2-year bioassay are presented below.

1 - Liver Tumors in B6C3F1 Mice

In the 2-year carcinogenicity bioassay in B6C3F1 mice, liver was the target tissue for neoplastic and non-neoplastic lesions in both males and females, as predicted by the hepatotoxicity observed in the subchronic study (NTP, 2011). The development of hepatic lesions is consistent with the dose-dependent liver toxicity reported in the subchronic study. Hepatocellular adenoma was observed at all doses starting at 37.5 mg/kg bw/day, whereas combined incidences of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma occurred with positive trends and were significantly increased in 75 mg/kg males and 150 mg/kg females. Osteomas and osteosarcomas and nasal metaplasia were also observed in a dose-dependent manner.

The hepatic neoplastic responses in male $B6C3F_1$ mice in the 2-year bioassay are not relevant to human risk for the following reasons:

a) They are consistent with the historically high levels of background hepatocellular neoplasms in this species and sex (Maronpot *et al.*, 1987), with 20-47% background incidences of combined hepatocellular adenoma and carcinomas (NTP, 2006) that more recently are reported to exceed 50% [e.g., 56% in the isoeugenol study (NTP, 2008) and 58% in the pulegone study (NTP, 2011)]. The limitations of 2-year mouse bioassays in susceptible strains of mice (e.g., B6C3F1) have been acknowledged also by the NTP with respect to their utility in extrapolating the results to human risk (Maronpot et al., 1987).

b) It is recognized that many liver tumors seen in 2-year bioassays in B6C3F1 mice are secondary to chronic toxicity and associated cellular proliferation as a function of dose. As an example, the status of p-nitrosodiphenylamine as a human carcinogen based on liver tumors reported in B6C3F1 mice was later revoked by the NTP (5th and 6th Annual Report on Carcinogens).

c) In the case of pulegone, toxicity is shown to be the result of the reactive menthofuran metabolites, which have been shown to bind to liver proteins and believed to be responsible for the hepatotoxicity of the parent compound, pulegone, and its primary metabolite menthofuran (McClanahan *et al*., 1989; Thomassen *et al*., 1992). Based on metabolic studies, the reactive metabolites are formed at high dose levels that are associated with significant toxicity. Given the low levels of human exposure and absence of chronic toxicity, these tumors are not considered relevant to human carcinogenicity risk (Cohen et al., 2004).

d) The validity of mouse hepatocellular tumors to predict human cancer risk has been questioned (Velazquez *et al*., 1996; Carmichael *et al*., 1997). Hepatocellular carcinoma is rare in humans, particularly as a result of chemical exposure. The major risk factors associated with liver tumors in humans are viral hepatitis, chronic and excessive alcohol consumption and exposure to aflatoxin, and in most cases tumors are accompanied by liver cirrhosis. Other non-genotoxic compounds that induce liver enzyme expression, such as phenobarbital, a known enzyme-inducer in the rodent liver, are also associated with rodent hepatocarcinogenicity. This bears no relevance to human risk given the long history of human use of such substances without increased risk of tumors in the liver or any other organ

in humans (McClain, 1990). This presents additional support for concluding that the rodent hepatocarcinogenicity is irrelevant for human risk assessment (Holsapple *et al*., 2006; Billington *et al*., 2010).

The conclusion that hepatocarcinogenesis in mice is not relevant to human cancer risk from pulegone intake has also been expressed by the European Food Safety Authority (EFSA) (EFSA, 2011) and by the National Industrial Chemicals Notification and Assessment Scheme of Australia (NICNAS, 2000).

2 - Bladder Papilloma and/or Carcinoma in Female Rats

In the 2-year carcinogenicity bioassay in F344/N rats, morbidity and mortality were observed at 75 mg/kg bw dose group of males and 150 mg/kg bw dose group of females, indicating severe toxicity at these dose levels, that also appeared to be irreversible, since morbidity and mortality remained high in these groups even after cessation of treatment at 60 weeks (stop-exposure treatment groups) (NTP, 2011). Morbidity and mortality were associated with the incidence and severity of kidney pathology (hyaline glomerulopathy, mineralization, chronic progressive nephropathy, congestion of the glomerulus) at the top two doses in both sexes. The only evidence of carcinogenicity involved small but significantly increased incidence of urinary bladder papilloma (3/47) and/or carcinoma (combined; 5/47) and only at the highest dose (150 mg/kg stop-exposure) in females that also experienced 100% mortality before the end of the study.

The urinary bladder neoplastic responses in female F344/N rats in the 2-year NTP bioassay are not relevant to human risk for the following reasons:

a) Urinary bladder papilloma and/or carcinoma were observed only at the highest dose (150 mg/kg stop-exposure) in females that also experienced 100% mortality before the end of the study. Treatment was discontinued at this dose level due to excessive morbidity and early mortality, indicating that this dose clearly exceeded a Maximum Tolerated Dose (MTD). As noted, the validity and biological significance of evidence of carcinogenesis under conditions of high levels of toxicity is questionable at best.

b) The observation of urinary bladder tumors in female but not in male rats provides further support for lack of direct carcinogenic potential of pulegone in the urinary bladder and is expected given that i) only female rats received the 150 mg/kg bw dose, while male rats received up to 75 mg/kg bw due to severe nephrotoxicity, ii) reactive and toxic metabolites are formed at high dose levels, above 80 mg/kg bw (Chen et al., 2001) and iii) even though the metabolite profile is the same between the sexes, urinary excretion of pulegone and/or menthofuran metabolites is approximately 40% higher in females compared to males across dose levels and slightly higher following repeated dose compared to equal single dose administration (Chen et al., 2001, 2003a). Therefore, the female urinary bladder was exposed to higher concentrations than the male bladder, thus explaining the more prominent bladder toxicity seen in females in the 2-year bioassays.

c) The urinary bladder lesions are the result of cytotoxicity and tissue regenerative responses, as demonstrated in a 6 week oral toxicity study in female F344/N that specifically investigated the tissue histological changes following administration of pulegone at 75 and 150 mg/kg bw/day (Da Rocha *et al*., 2012). Indeed, the administered dose was high enough for toxic metabolites to be formed. Four major metabolites were detected in the urine at both doses at levels determined to be cytotoxic *in vitro* [pulegone (0.36 and 0.46 mM), piperitone (0.50 and 0.41 mM), pipertenone (0.93 and 1.15 mM), and menthofuran (0.11 and 1.41 mM)] (Da Rocha *et al*., 2012). The urinary concentrations of pulegone and its metabolites in rats treated at both dose levels were higher than the urothelial cell LC_{50} or within the range of cytotoxic concentrations, as determined in a parallel *in vitro* study in human (1T1 cells) and rat (MYP3 cells) urothelial cell lines (Da Rocha *et al*., 2012).

d) Although no hyperplasia was detected in the bladders by light microscopy (or in the kidneys) at 4 weeks (time too short for the progression of the lesions), significant proliferative response, indicated by increased BrdU labeling index, in the bladder in the high-dose group showed a dose-related progression in surface SEM classification (Cohen *et al.*, 1990), with numerous small foci of superficial urothelial cell death (class 3), or extensive superficial urothelial cell death, especially in the dome of the bladder (class 4) that are considered precursors of a later tissue neoplastic response. Specifically, the proliferative index increased at a dose higher than the dose at which cytotoxicity was documented, indicating that proliferation was a tissue repair response subsequent to extensive cytotoxicity and epithelial cell loss. A continuum of dose-related benign or malignant tumors was observed in the 2-year bioassay (papillomas and carcinomas), consistent with progression from tissue remodeling to malignancy.

e) Therefore, bladder tumors in the urinary bladder of female rats treated with pulegone for 2 years are the result of a non-genotoxic mode of action characterized by series of progressive events, including a) chronic exposure to high concentrations, b) urinary excretion and concentration in the bladder of pulegone and/or cytotoxic metabolites, c) urothelial cytotoxicity and cell death, d) sustained regenerative tissue response to cell loss (urothelial cell proliferation) and e) urothelial tumor development, as a function of time (Da Rocha *et al*., 2012). On the basis of absence of convincing evidence of genotoxicity *in vitro* and particularly absence of genotoxicity *in vivo,* along with documented evidence of a non-genotoxic mode of action in the bladder of female rats, bladder tumors are secondary to dose-dependent cytotoxicity and consistent with a threshold of toxicity mode of action. The levels administered to rats, either in the NTP 2-year bioassay or the Da Rocha *et al*. (2012) study, exceed those possible for human intake. Since pulegone is self-limiting due to noxious sensation leading to aversion, intake levels that exceed a threshold of toxicity seen in the rat studies is highly improbable and the pathology observed in the female rat at high doses is therefore not relevant to human consumption of pulegone as a flavor ingredient.

G – Additional evidence for the safety of pulegone as a food flavor is derived from being a naturally occurring substance in several food sources and from its long history of safe use.

a) Pulegone is a naturally occurring compound in a wide variety of foods, herbs and oils at concentrations spanning a wide range. Pulegone is also a component of peppermint oil at concentrations up to *ca.* 0.2 -1.2%.

b) Pulegone is listed as a flavoring agent for direct addition to food at 21 CFR Part 172.515, and was determined to be generally recognized as safe (GRAS) by the FEMA Expert Panel in 1965. The FEMA Expert Panel reaffirmed the FEMA GRAS status of pulegone in 1996 (Adams et al., 1996). It has been reviewed for its safety as a flavoring agent by the Joint FAO/WHO Expert Committee on Food Additives and it was concluded to present no safety concern at current levels of intake and, therefore, assignment of an ADI was determined unnecessary (JECFA, 2000).

c) Pulegone is used at low levels (the average usual use levels range between 7-30 ppm[1]) and due to its strong organoleptic properties its use is self-limiting.

Conclusion

Pulegone does not present a carcinogenicity risk to humans and should not be listed in a future RoC. Claimed evidence of carcinogenicity in rodents is derived from studies conducted under conditions of questionable scientific validity on the basis of inappropriate dose range criteria. The tumors reported in rodents were observed only at dose levels exceeding the MTD and the overall metabolic and toxicity profile of pulegone demonstrates a mechanism of tumor development secondary to significant toxicity. Taken together with the absence of convincing evidence of mutagenicity of pulegone *in vitro* and particularly the absence of genotoxicity *in vivo*, there is no valid or scientifically defensible evidence of carcinogenesis even in the rodent models, under the criteria and definitions of published Cancer Risk Assessment Guidelines (US EPA, 2005).

We appreciate the opportunity to provide information relevant to the proposed listing of pulegone in a future edition of the RoC. We would be pleased to respond to any questions or comments that you may have.

Sincerely,

[Redacted]

Christie L. Harman, MPH
FEMA Acting Scientific Director

[1] Use in chewing gum applications may be higher due to retention of the flavoring substance in the gum base.

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