Essential oils from mint plants, including peppermint and pennyroyal oils, are used at low levels as flavoring agents in various foods and beverages. Pulegone is a component of these oils. In a 2-year bioassay, oral administration of pulegone slightly increased the urothelial tumor incidence in female rats. We hypothesized that its mode of action (MOA) involved urothelial cytotoxicity and increased cell proliferation, ultimately leading to tumors. Pulegone was administered by gavage at 0, 75, or 150 mg/kg body weight to female rats for 4 and 6 weeks. Fresh void urine and 18-h urine were collected for crystal and metabolite analyses. Urinary bladders were evaluated by light microscopy and scanning electron microscopy (SEM) and bromodeoxyuridine (BrdU) labeling index. Pulegone and its metabolites, piperitenone, piperitone, piperitone, menthofuran, and menthone, were tested for cytotoxicity in rat (MYP3) and human (1T1) urothelial cells by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. No abnormal urinary crystals were observed by light microscopy. Urine samples (18-h) showed the presence of pulegone, piperitone, piperitenone, and menthofuran in both treated groups. By SEM, bladders from treated rats showed superficial necrosis and exfoliation. There was a significant increase in the BrdU labeling index in the high-dose group. In vitro studies indicated that pulegone and its metabolites, especially piperitenone, are excreted and concentrated in the urine at cytotoxic levels when pulegone is administered at high doses to female rats. The present study supports the hypothesis that cytotoxicity followed by regenerative cell proliferation is the MOA for pulegone-induced urothelial tumors in female rats.

Key Words: pulegone; cytotoxicity; urinary bladder; mode of action; cell proliferation; metabolites.

Essential oils derived from many plants contain pulegone and are used at low levels for flavoring foods, drinks, and dental products (National Toxicology Program [NTP, 2011]). Pulegone, a monoterpene ketone, is a major constituent of pennyroyal oil that has also been used as a fragrance agent and as an herbal medicine to induce menstruation and abortion (Thomassen et al., 1990). In humans, case reports show that ingestion of high doses of pennyroyal oil is associated with gastritis, seizures, hepatic and renal effects, central nervous system toxicity, and coma (NTP, 2011).

In a 2-year bioassay (NTP, 2011), administration of pulegone to male and female IOFIII F344/N rats and B6C3F1 mice by corn oil gavage 5 days/week showed high morbidity and mortality at high doses in rats and decreased body weight gains in rats and mice. Effects in the kidneys (hyaline glomerulopathy and nephropathy), liver (oval cell hyperplasia, bile duct hyperplasia, hypertrophy, hepatocyte necrosis, and portal fibrosis), nose (olfactory epithelium degeneration, inflammation, and metaplasia), and forestomach (inflammation, hyperplasia, and ulcer) were reported. Increased incidences of liver neoplasms in male and female B6C3F1 mice in the study by NTP (2011) led to the conclusion that there was clear evidence of carcinogenic activity in mice. For female F344/N rats, it was concluded that there was some evidence of carcinogenicity based on an increased incidence of urinary bladder neoplasms. Five of 47 rats in the 150 mg/kg female stop-exposure group (gavage with pulegone stopped at week 60 because of severely reduced body weights) were diagnosed with papilloma and carcinoma, combined. Male rats did not show increased incidences of bladder tumors or neoplasms of other organs.

Pulegone was not mutagenic for Salmonella typhimurium (TA1537, TA98, TA1535, and TA100) with or without S9 activation (Andersen and Jensen, 1984). Pulegone was reported as mutagenic with rat liver S9 activation for S. typhimurium TA98 and Escherichia coli WP2 uvrA/pK M101 (NTP, 2011). However, in two other independent studies (NTP, 2011), pulegone was not mutagenic with or without activation (S. typhimurium TA97, TA98, TA100, and TA1535 and E. coli WP2uvrA/pKM101). The reasons for the discrepancy between tests by NTP (2011) are unknown as test protocols...
and concentrations for the studies were similar. *In vivo*, no significant increases in the frequency of micronucleated erythrocytes in a 3-month mouse study were found in the peripheral blood (NTP, 2011).

For a non–DNA-reactive carcinogen, tumors are induced by increasing urothelial cell proliferation (Cohen and Ellwein, 1990, 1991). This can be produced by either direct mitogenesis (propoxur is the only known example for the bladder) or more commonly by urothelial cytotoxicity and regenerative proliferation. Urothelial cytotoxicity can be due to urinary excretion of reactive chemicals (and/or metabolites) or the presence of urinary solids (Cohen, 1998; Cohen et al., 2007). Examination of the urine assesses the possibility of solids, although the presence of urinary solids is unlikely for pulegone. Metabolism studies on pulegone in rodents have identified 14 metabolites in urine, the major route of excretion of pulegone (Chen et al., 2001, 2003). The metabolic profile of pulegone is complex, with at least three pathways involved, including hydroxylation, reduction, or conjugation with glutathione as the first step (Chen et al., 2003). Piperitone, pipertenone, menthofuran, and menthone are among the metabolites that have been identified (Chen et al., 2003; Ferguson et al., 2007; Madyastha and Gaikwad, 1998). Pulegone itself was not detected in the urine of mice or rats after single or multiple doses in the study by Chen et al. (2003). However, Madyastha and Gaikwad (1998) reported that pulegone was detected in rat urine, and pipertenone was found to be the major metabolite. In humans, urine samples of six volunteers were collected 24 h after ingestion of 1000 μg of (S)-(+)-pulegone and 500 μg (R)-(+)-pulegone. The major metabolite of (R)-(+) pulegone was 10-hydroxypulegone. Only a trace amount of menthofuran was found and was not considered by the author to be a relevant metabolite in humans exposed to low doses of pulegone (Engel, 2003; NTP, 2011).

We hypothesized that the mode of action (MOA) for pulegone-induced rat bladder tumors involves cytotoxicity and regenerative cell proliferation. The objectives of this study were to determine the effects of oral gavage administration of pulegone on the urine and bladder epithelium of F344/N rats to provide data regarding its MOA for bladder tumor production, evaluate urinary concentrations of pulegone and major metabolites, and compare these urinary concentrations (pulegone and its four metabolites: menthofuran, menthone, pipertone, and pipertenone) with cytotoxic levels *in vitro* for human and rat urothelial cell lines.

**MATERIALS AND METHODS**

**In Vivo**

**Test material.** (R)-(+) Pulegone, CAS 89827 (97.5% purity), was purchased from Sigma-Aldrich (St Louis, MO), stored refrigerated, and protected from the light. For the purpose of dose formulations, the purity was assumed to be 100%. The test material was administered by oral gavage at concentrations of 75 and 150 mg/kg/day in corn oil (Sigma-Aldrich). The 150 mg/kg body weight/day dose level was selected as this was the high dose used in the carcinogenicity study (NTP, 2011). This dose was the only dose to produce a detectable incidence of female rat urinary bladder papillomas and carcinomas (combined). The 75 mg/kg body weight/day dose was selected because this was the next highest dose used in the carcinogenicity study, and no urothelial effects were observed at this dose in the 2-year bioassay. The dose volumes were based on body weights measured on the day of dose calculation and a dose factor of 10.0 ml/kg. All dose preparations were made every 28 days and stored refrigerated, protected from light, and sealed with paraffin.

**Study design.** Pulegone doses, administration schedule, diet, sex, and rat strain used were the same as in the 2-year bioassay by NTP (2011). Sixty 6-week-old female F344/N rats were purchased from Charles River Laboratories, Inc. (Portage, MI) and quarantined for 1 week. On arrival, the animals were placed in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study protocol was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC), and the level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 86-23, revised 1996). The animals were housed five per cage in polycarbonate cages with dry corn cob bedding in a room with a targeted temperature of 22°C, humidity of 50%, and light/dark cycle of 12 h (0600 lights on/1800 lights off, CST). Throughout the study, animals were fed powdered irradiated NTP-2000 (Zeigler Bros, Gardness, PA), the same as that used in the NTP bioassay. Diet and water were available *ad libitum* throughout the study. Nylabones (Nylabone Products, Neptune, NJ) were added to the cages for environmental enrichment. Following quarantine, animals were randomized into three groups (20 rats per group) using a weight stratification method (Martin et al., 1986). Rats were treated by oral gavage 5 days/week in the morning (approximately 0900 h–1100 h) with pulegone at a dose of 0 (group 1), 75 (group 2), or 150 (group 3) mg/kg body weight. Body weights were measured weekly, and water and food consumptions were measured during week 3. Detailed clinical observations were performed during study weeks 1 and 3.

Freshly voided urine was collected during study week 3 prior to gavage administration (approximately 0700–0900, CST) for determination of urinary pH immediately after collection by microelectrode (Microelectrodes Inc., Bedford, NH) and examination of the urinary sediment by light microscopy for the presence of crystals. During study week 6, urine was collected on ice from five animals per group over a period of approximately 18 h (1400–0830, CST). Animals were acclimated to metabolism cages for 24 h before urine collection. Food and water were available *ad libitum* during acclimation and during urine collection (Cohen et al., 2007). Urinary volume and creatinine (Beckman Coulter DxC 800; Beckman Coulter Inc., Brea, CA) were determined. Pulegone metabolites were identified and quantitated using an Agilent 7890 Gas Chromatograph (Santa Clara, CA) equipped with a flame ionization detector. Results were calculated using a three-point linear regression ranging from 3 to 200 μg/ml.

After treatment for 4 or 6 weeks, 10 animals from each group were sacrificed by an overdose of Nembutal (150 mg/kg body weight; Lundbeck, Deerfield, IL). Since changes found at the 4-week time point were considered sufficient for the purpose of this study, target tissues were collected from the animals treated for 6 weeks and appropriately preserved but were not examined. All animals were injected with bromodeoxyuridine (BrDU) 60 min prior to necropsy. At necropsy, bladders were inflated in situ with Bouin’s fixative and removed along with a small section of duodenum and placed in Bouin’s fixative. The duodenum served as a positive control to assess the adequacy of the BrDU injection. Kidneys and liver were removed, weighed, and fixed in 10% buffered formalin. Following fixation, bladders were bisected longitudinally, rinsed in 70% ethanol, and weighed. One-half of the bladder collected after treatment for 4 weeks was processed for scanning electron microscopy (SEM) (Cohen et al., 2007). Bladder halves collected after treatment for 6 weeks were placed in 70% ethanol but not processed further for SEM. Bladders were classified by SEM examination in one of five categories as previously described (Cohen et al., 1990). Briefly, class 1 bladders have flat, polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial...
urothelial cell death; class 3 bladders have numerous small foci of superficial urothelial cell death; class 4 bladders have extensive superficial urothelial cell death, especially in the dome of the bladder; and class 5 bladders have extensive cell death and piling up (hyperplasia) of rounded urothelial cells.

The other half of each bladder collected at both time points was cut longitudinally into strips and, with a slice of duodenal tissue, was embedded in paraffin. One section of liver and sections from both kidneys were also embedded in paraffin. Approximately 4-5 μm sections of the paraffin-embedded tissues collected after treatment for 4 weeks were stained with hematoxylin and eosin (H&E) and examined histopathologically (Cohen, 1983; Cohen et al., 1990, 2007). A diagnosis of mild simple hyperplasia in the bladder was made when there were four or more cell layers in the bladder epithelium.

Unstained slides of the bladder and intestinal tissue collected after treatment for 4 weeks were used for immunohistochemical detection of BrdU (Cohen et al., 2007). The duodenal tissue served as a positive control. Anti-BrdU (Millipore Corporation, Temecula, CA) was used at a dilution of 1:2000. If the intestinal tissue showed positive staining of the mucosa, the number of BrdU-labeled cells in at least 3000 urothelial cells (all layers) in the urinary bladder sections was counted to determine a labeling index (Cohen et al., 1997) and expressed as the percent of labeled cells to total number of cells.

**Statistics.** All values are reported as the mean ± SE. Group means for body and bladder weights, food and water consumptions, and the BrdU labeling index were evaluated using ANOVA, followed by Duncan’s multiple range test for group wise comparisons. Histopathology was compared using the two-tailed Fisher’s exact test. SEM data were analyzed using one-way nonparametric procedures followed by a chi-square test. P values less than 0.05 were considered significant. The statistical analyses were performed using SAS for Windows (Version 9.1).

**In Vitro**

**Test materials.** Pulegone (Sigma Aldrich) and selected metabolites in rat urine were tested for their relative cytotoxicity to rat and human urothelial cells in vitro. Piperitenone (purity 87.9%) was received as a gift from Nippon Terpene Chemicals Inc. (Tokyo, Japan). Piperitone (purity 94.3%) was received as a gift from Givaudan Schweiz AG (Dubendorf, Switzerland), and (−)-menthofuran (purity 99%) and menthone (purity 99.2%) were obtained from Sigma-Aldrich (Fig. 1). All test materials were stored at approximately 4–8°C in airtight containers, protected from light.

**Urothelial cell lines.** The MYP3 rat urothelial cell line and the 1T1 human urothelial cell line were provided by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL) and were used to assess the cytotoxic effects of the test substances on urothelial cells. The MYP3 cell line was obtained from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (MNU) (Kawamata et al., 1993). The cell line has retained the characteristics of epithelial cells in culture, expresses keratin 5 messenger RNA, does not exhibit anchorage-independent growth, and does not cause development of tumors in nude mice. The 1T1 cell line was derived from normal human ureter epithelium and immortalized by transfection of the HPV-16 E6 and E7 genes (Tamatani et al., 1999).

**Cell culture conditions.** MYP3 cells were grown in Ham’s F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 10 μM nonessential amino acids, 10 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 5 μg/ml transferrin, and 100 U/ml penicillin and 100 μg/ml streptomycin (supplied as a penicillin and streptomycin mixture) (all from Gibco), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 250 mg/ml dextrose and 1 mg/ml hydrocortisone from Sigma (St Louis, MO). 1T1 cells were cultured in Keratinocyte-SFM (1x) supplemented with bovine pituitary extract (25 mg minimum) and human recombinant EGF (2.5 μg minimum) (Gibco-BRL). All cells were grown at 37°C in 5% CO₂.

**Evaluation of cytotoxicity.** The cytotoxicity of pulegone and its metabolites on MYP3 and 1T1 cells was assessed by determining viability. Pulegone and its metabolites were dissolved in dimethyl sulfoxide (DMSO) prior to diluting in the appropriate medium for the cell line to the concentrations used for testing. Only working solutions with concentrations of DMSO ≤ 0.172% were used. Each concentration was tested in four wells. MYP3 cells were seeded at a concentration of 4000 cells per well and 1T1 cells were seeded at a concentration of 6000 cells per well into 96-well plates. Treatment began 24 h after seeding and continued for 3 days with no change in medium. At the end of treatment, cell viability was determined by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. For each plate, the % survivability for each treated well was calculated as the ratio of the mean absorbance of the treated wells to the mean absorbance of the negative control (untreated cells). The mean ± SE from all experiments for each chemical were subjected to nonlinear regression analysis to determine the LC₅₀. Nonlinear regression analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

**RESULTS**

**In Vivo**

**Animal survival and clinical observation.** There were no instances of treatment-related animal morbidity or mortality in this study. One animal in the control group died following gavage administration. The necropsy evaluation of this animal showed an oily residue in the lungs, most likely the dosing solution. On study day 4, bloody nasal mucus was noted in various animals from all study groups. Since pulegone is a highly volatile substance (vapor pressure 138 mm Hg at 25°C) and the animal room had a mint smell, we suspected that volatilization of the substance irritated the nose of the rats. Pulegone is known to induce nose lesions (NTP, 2011). Consequently, filter tops were placed on the cages and the treatment solution was covered with parafilm during mixing and gavage treatment. The parafilm was opened only when filling the syringe with the dose volume. After these changes, a considerable improvement in the condition of the rats was observed, especially in the control rats.

During the experiment, treated rats showed alopecia around the mouth and yellow staining and alopecia around the urogenital area. These observations were treatment related and were more evident in the high-dose group.
Body weights. The mean body weights of rats treated with 150 mg/kg pulegone were significantly lower compared with control groups from study day 6 onward. The animals treated with 150 mg/kg pulegone gained less weight during the experiment compared with the 75 mg/kg pulegone and control groups (data not shown). There was no difference in body weights between the 75 mg/kg pulegone group and the controls.

Food and water consumptions. There was no difference in food consumption between groups. The water consumption, measured as grams per rat per day and grams per kg body weight per day was increased in the 75 and 150 mg/kg pulegone-treated groups and was statistically different compared with control for both parameters (data not shown).

Body and tissue weights at necropsy. At the first sacrifice, after 4 weeks of treatment, terminal body weights of the 150 mg/kg pulegone group showed a significant decrease compared with the control group. There was no difference in bladder weights of the pulegone-treated groups compared with the control group. There was a statistically significant increase in the relative kidney weight in the 75 and 150 mg/kg pulegone groups compared with the control group. The absolute and relative liver weight was significantly increased in the high-dose pulegone group compared with the control group (Supplementary table S1).

After 6 weeks of treatment, final body weights in the 150 mg/kg pulegone-treated rats showed a significant decrease compared with the control group. Relative bladder and kidney weights and absolute and relative liver weights were significantly increased in the 150 mg/kg pulegone group compared with the control group (Supplementary table S2).

Histopathological examination of kidneys, liver, and urinary bladder. After treatment for 4 weeks, there was no significant incidence of changes in the bladder epithelium of pulegone-treated groups by light microscopy. No histological changes were found in the kidneys of the animals. Mild to moderate single-cell necrosis was observed in all livers in the 150 mg/kg group. The liver was normal in the control and 75 mg/kg groups.

Immunohistochemical determination of BrdU. There was a significantly increased BrdU labeling index in the 150 mg/kg pulegone group compared with the control. In the 75 mg/kg pulegone treatment group, the labeling index was slightly increased compared with the control group, but the increase was not statistically significant (Table 2).

SEM examination of the bladder epithelium. The SEM analysis showed a gradual dose related increase in the classification of the bladder surface (Table 2). There was evidence of cytotoxicity and necrosis of the superficial cell layer in the 75 and 150 mg/kg pulegone treatment group (Figure 2). In the 0 mg/kg pulegone control group, one bladder was class 3, but no bladders were class 4 or 5. In the 75 mg/kg pulegone group, three class 3 bladders and two class 4 bladders were observed. In the 150 mg/kg pulegone treatment group, three bladders were class 3 and four bladders were class 4, significantly different from control (p < 0.05) (Table 2). The severity of the lesions increased with an increase in the dose administered.

TABLE 1
Comparison of In Vivo Urinary Concentration and In Vitro Cytotoxicity

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>In vivo urinary concentration (mM)</th>
<th>In vitro cytotoxicity (LC50 in mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75 mg/kg</td>
<td>150 mg/kg</td>
</tr>
<tr>
<td>Pulegone</td>
<td>0.36 ± 0.11</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>Piperitenone</td>
<td>0.93 ± 0.28</td>
<td>1.15 ± 0.15</td>
</tr>
<tr>
<td>Piperitone</td>
<td>0.50 ± 0.12</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>Menthofuran</td>
<td>0.11 ± 0.02</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>Menthone</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. ND, not detected.

*Pulegone dose administered to the rat.
Determination of LC50 for IT1 cells. The results are reported in the same manner as described for MYP3 cells. The LC50 for pulegone, piperitenone, piperitone, (+)-menthofuran, and menthone was 0.57, 0.44, 1.29, 3.60, and 7.25 mM, respectively (Table 1).

**DISCUSSION**

The 150 mg/kg pulegone concentration, administration schedule, diet, and the rat strain used in this study were the same as in the 2-year bioassay that demonstrated a weak tumorigenic potential for pulegone on the female IOFIIle F344 rat urinary bladder (NTP, 2011). The 75 mg/kg dose was the next highest dose used in the 2-year bioassay but showed no effect on the bladder epithelium in that study.

The clinical findings in the present study included decreased body weight gain, bloody nasal mucous, and alopecia around the mouth and urogenital area. Decreased body weight gain is a common observation in pulegone gavage studies. In the NTP 2-year study, rats and mice of both genders that received 75 and 150 mg/kg had at least a 10% decreased body weight gain.

### TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Histopathology</th>
<th>BrdU labeling index (%), mean ± SE (n)</th>
<th>SEM classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Simple hyperplasia</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 mg/kg pulegone</td>
<td>10</td>
<td>0</td>
<td>0.03 ± 0.02 (10)</td>
</tr>
<tr>
<td>2</td>
<td>75 mg/kg pulegone</td>
<td>9</td>
<td>1</td>
<td>0.09 ± 0.05 (7)</td>
</tr>
<tr>
<td>3</td>
<td>150 mg/kg pulegone</td>
<td>10</td>
<td>0</td>
<td>1.13 ± 0.18 (8)*</td>
</tr>
</tbody>
</table>

*SEM classification significantly different from 0 mg/kg pulegone group, p < 0.05.

**FIG. 2.** SEM—(A) control group. Normal rat bladder showing large flat polygonal superficial cells (×400; bar = 100 μm). (B) Pulegone 75 mg/kg group. Rat bladder showing necrosis and exfoliation (×629; bar = 100 μm). (C) Pulegone 150 mg/kg group. Rat bladder showing extensive area of necrosis and exfoliation (×600; bar = 100 μm). (D) Pulegone 150 mg/kg group. Rat bladder showing loss of superficial cell layer (×800; bar = 50 μm).
compared with the control vehicle group (NTP, 2011). Since food consumption was the same in all groups of this study, the decreased body weights could be considered a treatment-related toxic effect of pulegone administration. Pulegone treatment increased water consumption accompanied by increased urine volume, which resulted in lower creatinine concentrations in the urine compared with control.

After 4 weeks of treatment, SEM analysis showed extensive urothelial superficial cell necrosis and exfoliation in the treated groups, especially in the 150 mg/kg pulegone group. Two bladders in the 75 mg/kg and four bladders in the 150 mg/kg pulegone treatment groups were defined as class 4. Although the changes in the 75 mg/kg group were limited, it is likely that they represent a treatment-related effect since the urinary concentrations of pulegone and some of its metabolites were greater than concentrations required to produce cytotoxicity. The urinary concentrations were actually similar at the two administered doses, suggesting that metabolism was nearly saturated at the 75 mg/kg dose. Detection of superficial necrosis by SEM without detection by light microscopy is common (Cohen et al., 2007). SEM examination allows visualization of events not detectable by light microscopy and also has the added benefit of providing vastly more of the bladder luminal surface for examination in addition to being more sensitive and specific for the evaluation of the urothelium for superficial cytotoxicity. The SEM results demonstrate that cytotoxicity of the bladder surface was induced by pulegone treatment in female rats. Class 4 changes (severe and extensive necrosis) are not seen in control rats. Such a low incidence of cytotoxicity of the urothelium is common for chemicals having a low incidence of bladder tumors in a long-term bioassay (Cohen et al., 2007). Furthermore, the BrdU labeling index was greatly increased in the 150 mg/kg pulegone groups, clearly indicating a significant proliferative response.

Increased cell proliferation occurred consequent to urothelial cell death, as was demonstrated in the present study by a marked increase in the BrdU labeling index in the high-dose pulegone group. The mean BrdU labeling index (%) in the 75 mg/kg group was 0.09 ± 0.05, slightly greater than controls (0.03 ± 0.02) but not statistically significant, but in the 150 mg/kg, it was significantly different from the control group. Increased proliferation of the urothelium occurs either due to direct mitogenesis, with only one known example for the bladder (propoxur) (Cohen et al., 1994) or more commonly is due to increased cell death and regeneration (Cohen, 1998). Increased cell death in the urinary bladder is usually due to necrosis. Regenerative proliferation ensues which is dependent for its continuance on sustained exposure to the inciting stimulus. Our results showed that pulegone induced urothelial cytotoxicity and necrosis with consequent increased cell proliferation. The continued cell proliferation leads eventually to urothelial hyperplasia, and after long-term exposure, it leads to tumors (Cohen, 1998). In the present short-term study, there was no increased incidence of urothelial hyperplasia despite the significant necrosis and increased rate of proliferation. This suggests that the urothelial cells do not accumulate in number due to necrosis of regenerated cells, a balance between cell deaths and cell births.

In addition to a slightly increased incidence of urinary bladder neoplasms at 150 mg/kg, findings in the NTP report included dose-related increases in the incidence of non-neoplastic lesions in the nasal epithelium of male and female mice, and one 150 mg/kg male mouse and one 75 mg/kg female mouse developed a nasal osteoma. The NTP report concluded that it was unlikely that pulegone volatilization increased the exposure. They concluded that these changes were associated with high levels of xenobiotic metabolizing enzymes present in the nasal epithelium (NTP, 2011). However, pulegone itself appears to be cytotoxic to epithelial cells, at least urothelial cells in the present experiments. More likely, it is a combination of increased exposure from volatilization and metabolism by the nasal mucosa that contributes to the nasal effects indicative of contact cytotoxicity. Pulegone is highly volatile as evidenced in the present study by the presence of effects in control rats until microisolator covers were placed on the cages along with other preventive measures.

Cytotoxicity and necrosis of the urothelium can be caused by urinary solids, cytotoxic chemicals (and/or metabolites), or extremes of urinary composition, such as pH > 9 (Cohen, 1998). Light microscopic evaluation of fresh void urine collected on day 19 in the present study showed no abnormal or increased crystals. It is unlikely that changes in urinary solids or pH play a role in the MOA of pulegone on the rat urinary bladder.

Analysis of 18-h urines collected during study week 6 showed that piperitenone had the highest concentration of the chemical and metabolites, followed by piperitone, pulegone, and menthofuran. In another study, rats treated with 250 mg/kg pulegone showed the same metabolites, with piperitenone also in highest concentration. In addition, p-cresol, benzoic acid, and 5-OH-pulegone were also found (Madyastha and Gaikwad, 1998). Multiple studies from the NTP identified 14 different metabolites in rats treated with pulegone in single or multiple doses; however, no pulegone was detected in the urine (Chen et al., 2001, 2003). Differences in findings could be due to time of pulegone treatment, dose, methods employed for metabolite detection, and specifically methods for urine collection. In the studies by Chen et al. (2001, 2003), collection of urine was performed at room temperature, whereas in our study and in those reported by Madyastha and Gaikwad (1998), urine was collected on ice to avoid chemical volatilization or degradation. Since pulegone and some of its metabolites are highly volatile, it is likely that collection of samples at room temperature resulted in loss of much or all of these chemicals. The complete scheme of pulegone metabolism in rats remains unclear.

In our in vitro study, piperitenone and pulegone showed the lowest LC50 concentrations in rat and human urothelial cell lines, followed by piperitone, menthofuran, and menthone. Pulegone concentrations found in the urine of rats treated with 75 and 150 mg/kg pulegone were higher than the LC50s found...
in the rat urothelial cell line. Piperitenone had the lowest LC$_{50}$ of the metabolites tested and was the major metabolite in the urine. The mixture of pulegone and some of its metabolites is the likely cause of the urothelial cytotoxicity that was detected, especially piperitenone and pulegone, which were present in the urine at concentrations greater than the LC$_{50}$ concentration for urothelial cells detected in vitro. The menthofuran concentration detected in rat urine was lower than the LC$_{50}$ found for human and rat urothelial cell lines but at a level that might have contributed slightly to the urothelial cytotoxicity. Our in vitro results indicate that pulegone and its metabolites, especially piperitenone and pulegone, are excreted and concentrated in the urine at cytotoxic concentrations when pulegone is administered at high doses to female rats. It is only when sufficiently high concentrations are attained in the urine that cytotoxicity can occur. Since pulegone and its metabolites are excreted predominantly in the urine (Chen et al., 2003) and concentrated, exposure is likely higher for the urothelium than for other tissues, suggesting an explanation for the target organ specificity for the tumor-inducing effect in rats.

The key events for the MOA for pulegone-induced urinary bladder tumors in female rats are (1) chronic exposure to high concentrations of pulegone; (2) metabolism, excretion, and concentration of pulegone and cytotoxic metabolites, especially piperitenone, in the urine; (3) urothelial cytotoxicity; (4) sustained regenerative urothelial cell proliferation; and (5) development of urothelial tumors. This MOA implies a threshold effect, requiring high exposure to pulegone to generate sufficiently high concentrations of pulegone and metabolites in the urine to produce cytotoxicity. Similar metabolites are likely produced and excreted in the urine in humans (Engel, 2003; NTP, 2011), and they have similar cytotoxic potential on the human urothelium (as evidenced by their effects on 1T1 cells in the present study). However, humans cannot be chronically exposed to such high levels of pulegone because of the severe nasal irritation and noxious sensation experienced with such high exposures. What dose levels are required to produce urinary concentrations that could induce cytotoxicity have not been determined but likely are within an order of magnitude or less of the 75 mg/kg dose based on toxicity studies performed by the NTP (2011). Taking into account the critical quantitative differences between long-term exposures in the rat bioassay and the potential for human exposure, the MOA for bladder tumor induction appears to not be relevant to humans. Sufficiently high chronic exposures, the initial key event in the MOA, cannot occur in humans because of the very noxious nature of the highly volatile pulegone.

Multiple studies in bacteria have shown that pulegone is not mutagenic with or without activation (Andersen and Jensen, 1984; NTP, 2011). In a single study, pulegone was mutagenic in two bacterial strains, but only with S9 activation (NTP, 2011). Menthone, a pulegone metabolite, has been reported as mutagenic, although only at the two lowest doses tested (Andersen and Jensen, 1984). However, we found no menthone in the urine of pulegone-treated rats. NTP concluded that pulegone is genotoxic based on the single positive result. We believe that this conclusion is not supported by the data available, especially without some evidence that DNA adducts are actually formed. The possible mode of pulegone-induced DNA reactivity in the target tissue, the urothelium, requires further investigation.

Nevertheless, the present studies support the hypothesis that the MOA for pulegone-induced tumorigenicity in female rats involves urothelial cytotoxicity followed by regenerative cell proliferation, ultimately leading to tumors. Pulegone administered to rats induced urothelial necrosis and increased urothelial cell proliferation. Analysis of urine from treated rats showed that pulegone and its metabolites were present in the urine at cytotoxic concentrations. It is unlikely that humans could be exposed to the exceptionally high concentration of pulegone necessary to generate the high urinary concentrations required to produce urothelial cytotoxicity.

SUPPLEMENTAL DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


