d-LIMONENE

This substance was considered by a previous working group, in 1992 (IARC, 1993). Since that time, new data have become available, and these have been incorporated into the monograph and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

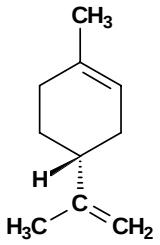
Chem. Abstr. Serv. Reg. No.: 5989-27-5

Chem. Abstr. Name: (R)-1-Methyl-4-(1-methylethenyl)cyclohexene

IUPAC Systematic Name: (R)-(+)-*para*-Mentha-1,8-diene

Synonyms: (+)-Dipentene; (R)-4-isopropenyl-1-methyl-1-cyclohexene; D-limonene; d-(+)-limonene; D-(+)-limonene; (+)-limonene; (+)-α-limonene; (+)-(R)-limonene; (+)-(4R)-limonene; (R)-limonene; (R)-(+)-limonene; (4R)-(+)-limonene; (+)-*para*-mentha-1,8-diene; (R)-*p*-mentha-1,8-diene; (R)-(+)-*para*-mentha-1,8-diene

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{16}$ Relative molecular mass: 136.24

1.1.3 *Chemical and physical properties of the pure substance*

(*a*) *Description*: Colourless liquid with characteristic citrus odour (National Toxicology Program, 1991)

(*b*) *Boiling-point*: 175.5–176°C (Budavari, 1996)

(*c*) *Melting-point*: –96.9°C (National Toxicology Program, 1991); –74.3°C has also been reported (Lide, 1991)

(*d*) *Density*: 0.8411 g/cm^3 at 20°C (National Toxicology Program, 1991)

(*e*) *Solubility*: Slightly soluble in water (13.8 mg/mL at 25°C); soluble in acetone, dimethyl sulfoxide and ethanol (National Toxicology Program, 1991; National Library of Medicine, 1998)

(*f*) *Volatility*: Vapour pressure, 133 Pa at 14°C and 665 Pa at 40.4°C; relative vapour density (air = 1), 4.69 (National Toxicology Program, 1991)

(*g*) *Conversion factor*: $mg/m^3 = 5.57 \times ppm$

1.2 Production and use

Information available in 1995 indicated that *d*-limonene was produced in Australia, Brazil, Germany, Japan and the United States (Chemical Information Services, 1995).

d-Limonene has been used for many years as a flavour and fragrance additive in foods, beverages and consumer products. It is increasingly used as a solvent. It is also used in the manufacture of resins, as a wetting and dispersing agent and in insect control (National Toxicology Program, 1991; IARC, 1993; Budavari, 1996).

d-Limonene and its metabolite perillyl alcohol are currently undergoing clinical trials for use in treatment of breast cancer and other tumours, and chemoprevention trials are under consideration (Gould, 1995; O'Shaugnessy, 1996; Vigushin *et al*., 1998).

1.3 Occurrence

1.3.1 *Natural occurrence*

d-Limonene is one of the most common terpenes in nature, occurring in citrus and a wide variety of other plant species. It is a major constituent of oil of citrus rind, dill oil, oil of cumin, neroli, bergamot and caraway (National Toxicology Program, 1991) [see IARC (1993) for a detailed discussion of its occurrence].

1.3.2 *Occupational exposure*

According to the 1981–83 National Occupational Exposure Survey (National Institute for Occupational Safety and Health, 1998), approximately 138 300 workers in the United States were potentially exposed to *d*-limonene. Occupational exposure to *d*-limonene may occur during its production and use, notably as an industrial solvent.

1.3.3 *Environmental occurrence*

The average daily dietary intake of *d*-limonene has been estimated to be about 0.3 mg/kg bw (Flavor and Extract Manufacturers' Association, 1991).

d-Limonene has been detected in indoor and outdoor air in various locations (IARC, 1993; National Library of Medicine, 1998).

1.4 Regulations and guidelines

The 8-h time-weighted exposure limit for *d*-limonene in Sweden is 150 mg/m^3 and the short-term (15 min) exposure limit is 300 mg/m^3 (National Board of Occupational Safety and Health, 1996).

No international guidelines for *d*-limonene in drinking-water have been established (WHO, 1993).

2. Studies of Cancer in Humans

No data were available to the Working Group.

3. Studies of Cancer in Experimental Animals

Previous evaluation

d-Limonene was tested for carcinogenicity by oral gavage in one study in mice and one study in rats. In mice and female rats, there were no treatment-related tumours, but male rats had renal tubular hyperplasia and a significantly increased combined incidence of renal tubular adenomas and carcinomas. In a two-stage experiment, oral treatment with *d*-limonene after administration of *N*-nitrosoethylhydroxyethylamine enhanced the development of renal tubular hyperplasia and renal tubular adenomas in male Fischer rats, which synthesize α_{2u} -globulin in the liver in large quantities, but not in male NBR rats, which do not (IARC, 1993). [The present Working Group reconfirmed the adequacy of the bioassays of *d*-limonene in male and female mice and rats conducted by the National Toxicology Program as cited in IARC (1993), noting the convincing renal tumour response in male rats and the absence of renal tumours in female rats.]

New studies

3.1 Administration with known carcinogens

Mouse: In a model of lung carcinogenesis, groups of 25 female A/J mice, six to seven weeks of age, were fed *d*-limonene in the diet at a concentration of 0.63% for 17 weeks. One week after commencement of treatment, the mice received an intraperitoneal injection of 10 μmol 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone (NNK) in 0.1 mL saline. *d*-Limonene treatment significantly reduced the number of lung tumours per mouse, from 8.1 in mice given NNK alone to 2.4 ($p < 0.05$), although the percentage of mice with lung tumours was not significantly different between those given NNK (100%) and those given *d*-limonene (96%) (El-Bayoumy *et al*., 1996).

Rat: In a model of putative colonic preneoplasia, groups of 12 male Fischer 344 rats, five weeks of age, were given 0.5% *d*-limonene in the drinking-water (equivalent to 0.67% in the diet) for five weeks. One week after the start of treatment, the rats received subcutaneous injections of 15 mg/kg bw azoxymethane once a week for three weeks and were killed at 10 weeks of age. In rats given azoxymethane and *d*-limonene, the frequencies of aberrant crypt foci and the numbers of aberrant crypts per focus and of aberrant crypts per colon were significantly decreased ($p < 0.01$ to $p < 0.001$) when compared with the group given azoxymethane alone (Kawamori *et al*., 1996).

In a multi-organ model of carcinogenesis, groups of 20 Fischer 344 male rats aged five weeks were treated sequentially with *N*-nitrosodiethylamine (100 mg/kg bw by intraperitoneal injection as a single dose at the beginning of the study), *N*-methyl-*N*-nitrosourea (20 mg/kg bw by intraperitoneal injection four times during weeks 1 and 2), *N*-nitrosobutyl-*N*-(4-hydroxybutyl)amine (0.05% in drinking-water during weeks 1 and 2), 1,2-dimethylhydrazine (40 mg/kg bw by subcutaneous injection four times during weeks 3 and 4) and *N*-nitrosodihydroxydipropylamine (0.1% in drinking-water during weeks 3 and 4). From the end of week 4, *d*-limonene at doses of 0.5, 1 or 2% was administered in the diet for 24 weeks, when surviving rats were sacrificed (i.e. 28 weeks after the start of the study). A group receiving 2% *d*-limonene but no initiating carcinogen schedule was also included. The number of rats with renal tubular adenomas was statistically significantly increased ($p < 0.01$) at the high dose of *d*-limonene: 13/19 with the carcinogen schedule plus 2% *d*-limonene and 4/19 with the carcinogen schedule alone. This increase in renal tumour incidence was accompanied by an increased number of atypical tubules (a preneoplastic lesion) per rat. None of the rats fed 2% *d*-limonene alone had a renal tumour. No modification of carcinogenesis was observed in any other organ (Kimura *et al*., 1996).

Hamster: In a model of pancreatic carcinogenesis, groups of 25 Syrian golden hamsters, five weeks of age, were given subcutaneous injections of 15 mg/kg bw *N*-nitrosobis(2-oxopropyl)amine in 0.9% saline once a week for five weeks and simultaneously fed chow pellets containing 0, 1 or 2% *d*-limonene for up to 26 weeks, when the animals were killed. The high dose of *d*-limonene significantly decreased the number of pancreatic carcinomas, from 0.74 ± 0.15 in the animals given the carcinogen alone to 0.25 ± 0.12 in the animals given carcinogen plus 2% *d*-limonene ($p < 0.05$) (Nakaizumi *et al*., 1997).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

d-Limonene is absorbed from the gastrointestinal tract. Two male volunteers given 1.6 g [^{14}C]*d*-limonene orally excreted 52–83% of the dose in their urine within 48 h. The major urinary metabolite isolated was 8-hydroxy-*para*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (Kodama *et al.*, 1976).

The toxicokinetics of *d*-limonene were studied in volunteers exposed by inhalation for 2 h to 10, 225 or 450 mg/m^3 *d*-limonene and to a workload of 50 W to simulate light physical activity; 10 mg/m^3 was considered to be the control concentration. The relative pulmonary uptake was approximately 70% of the amount supplied. Three linear phases of elimination could be distinguished in the time studied: a terminal phase of slope γ for slow elimination (320–1300 min after exposure), an intermediate phase of slope β for rapid elimination (16–319 min after exposure) and an initial phase of slope α (0–15 min after

exposure). The plasma half-life of *d*-limonene was approximately 2.6 min for the α phase, 32 min for the β phase and 75 min for the γ phase. The lung clearance rate in 4 h was 1.1 L/kg per h for 225 mg/m^3 and 1.4 L/kg per h for 450 mg/m^3, and the lung clearance rate in 21 h was 1.1 L/kg per h for 450 mg/m^3 (Falk-Filipsson *et al.*, 1993).

A pilot study was conducted in healthy volunteers (five women, two men) to investigate the metabolism and the toxicity of pharmacologically (supradietary) administered *d*-limonene. After the subjects had ingested 100 mg/kg *d*-limonene in a custard, their blood was drawn at 0 and 24 h for blood chemistry and at 0, 4 and 24 h for analysis of metabolites. Gas chromatography–mass spectrometry indicated the presence of five *d*-limonene metabolites in plasma: two major peaks were identified as dihydroperillic acid and perillic acid and a third major peak was limonene-1,2-diol; limonene itself was only a minor component. Two minor peaks were found to be the respective methyl esters of the acids. In all subjects, the metabolite concentrations were higher at 4 h than at 24 h, but a half-life value was not determined (Crowell *et al.*, 1994).

The toxicokinetics of *d*-limonene were studied in two women with breast cancer and one man with colorectal cancer. The patients received 0.5–12 g/m^2 body surface area per day orally for 21 days, and plasma and urine samples were collected on days 1 and 21. The metabolites were characterized and their structures elucidated by liquid chromatography–mass spectrometry and nuclear magnetic resonance spectrometry. Five major metabolites were detected in plasma: limonene-1,2-diol, limonene-8,9-diol, perillic acid, an isomer of perillic acid and dihydroperillic acid. The urinary metabolites comprised the glucuronides of the two isomers of perrilic acid, limonene-8,9-diol and a monohydroxylated limonene. The results are consistent with those of previously published studies in humans and in animals, but this study was the first in which limonene-8,9-diol and an additional isomer of perillic acid were identified (Poon *et al.*, 1996).

In a phase I clinical trial of orally administered *d*-limonene, 17 women and 15 men aged 35–78 (median, 57), with advanced metastatic solid tumours received an average of three treatment cycles of 21 days (one dose on day 1, then three daily doses on days 4–21) at doses ranging from 0.5 to 12 g/m^2 body surface area. *d*-Limonene was slowly absorbed, the maximal plasma concentration being attained at 1–6 h. The mean peak plasma concentrations of *d*-limonene were 11–20 μmol/L, and the predominant metabolites were perillic acid (21–71 μmol/L), dihydroperillic acid (17–28 μmol/L), limonene-1,2-diol (10–21 μmol/L), uroterpinol (14–45 μmol/L) and an isomer of perillic acid. After reaching these peaks, the plasma concentrations decreased according to first-order kinetics. The values for the integrated area under the curve for time–concentration showed little variation with administered dose. There was no accumulation of the parent or metabolites after a treatment cycle (Vigushin *et al.*, 1998).

4.1.2 *Experimental systems*

[^{14}C]*d*-Limonene was absorbed rapidly after administration by gavage of 800 mg/kg bw (4.15 μCi/animal) to male Wistar rats. The radiolabel concentration in blood was maximal after 2 h, and large amounts of radiolabel were also observed in the liver

(maximal after 1 h) and kidneys (maximal after 2 h). Negligible concentrations were found in blood and organs after 48 h (Igimi *et al.*, 1974).

Urinary recovery of [^{14}C]*d*-limonene was 77–96% within three days in rats, guinea-pigs, hamsters and dogs. Faecal recovery was 2–9% within three days (Kodama *et al.*, 1976). Bile-duct-cannulated rats given *d*-limonene orally excreted 25% of the dose in the bile within 24 h (Igimi *et al.*, 1974).

After oral administration of *d*-limonene to rabbits, the urinary metabolites isolated were *para*-mentha-1,8-dien-10-ol (M-I on Figure 1), *para*-menth-1-ene-8,9-diol (M-II), perillic acid (M-III), perillic acid-8,9-diol (M-IV), *para*-mentha-1,8-dien-10-yl-β-D-glucopyranosiduronic acid (M-V) and 8-hydroxy-*para*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (M-VI) (Kodama *et al.*, 1974). After oral administration of *d*-limonene to dogs and rats, five other urinary metabolites were isolated: 2-hydroxy-*para*-menth-8-en-7-oic acid (M-VII), perillylglycine (M-VIII), perillyl-β-D-glucopyranosiduronic acid (MIX), *para*-mentha-1,8-dien-6-ol (M-X) and probably *para*-menth-1-ene-6,8,9-triol (M-XI). The major urinary metabolite was M-IV in rats and rabbits, M-IX in Syrian hamsters, M-II in dogs and M-VI in guinea-pigs (Kodama *et al*., 1976). The possible metabolic pathways of *d*-limonene are shown in Figure 1.

Under alkaline extraction conditions, *d*-limonene was metabolized by rat liver microsomes *in vitro* to the glycols *d*-limonene 8,9-diol and *d*-limonene 1,2-diol *via* the 8,9- and 1,2-epoxides (Watabe *et al.*, 1980, 1981). Under neutral extraction conditions, no hydrolysis of *d*-limonene-1,2-epoxide to its corresponding diol was observed (Lehman-McKeeman *et al.*, 1989). In rat liver microsomes, expoxidation of *d*-limonene at the 1,2 double bond occurs only in the *cis* orientation, whereas in mouse liver microsomes both *cis* and *trans* isomers of this epoxide are formed (Lehman-McKeeman & Caudill, 1992a).

When [^{14}C]*d*-limonene was administered orally to male and female Sprague-Dawley rats at a dose of 409 mg/kg bw, the renal concentration of *d*-limonene equivalents was about 2.5 times higher in males than females, and approximately 40% of the radiolabel in male rat kidneys was bound reversibly to renal proteins. The major metabolite bound to the renal protein fraction was identified as *d*-limonene-1,2-epoxide (> 80%), parent *d*-limonene and the 1,2-diol representing minor components of the protein-bound moieties. The renal protein to which these metabolites bound was identified as α_{2u}-globulin by high-performance liquid chromatography (Lehman-McKeeman *et al.*, 1989).

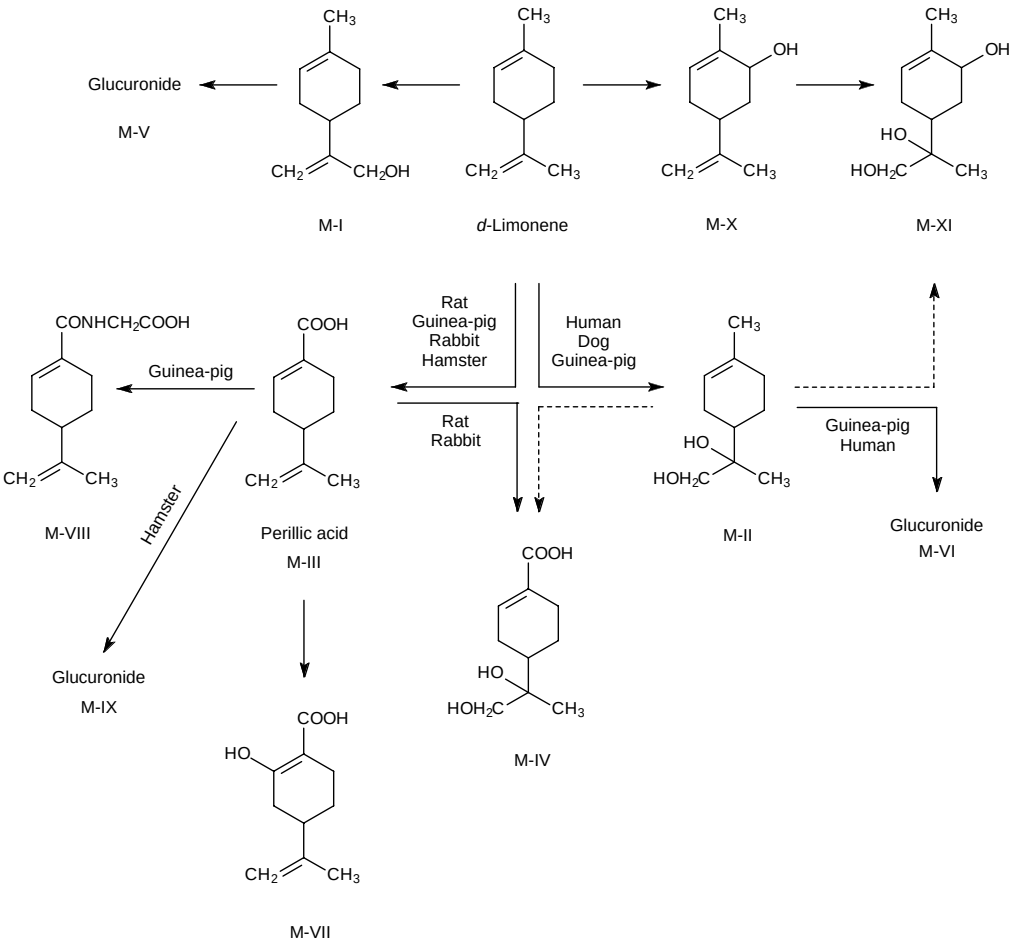
4.2 Toxic effects

4.2.1 *Humans*

Five healthy adult male volunteers who received a single oral dose of 20 g *d*-limonene all developed transient proteinuria, non-bloody diarrhoea and tenesmus. The results of functional tests of the liver, kidney and pancreas were normal (Igimi *et al.*, 1976).

In the study of volunteers treated by inhalation, described in section 4.1.1, no irritating or central nervous system-related symptoms were found. A 2% decrease in lung vital capacity was found after exposure to 450 mg/m^3 when compared with 10 mg/m^3; this difference was statistically but not clinically significant (Falk-Filipsson *et al.*, 1993).

Figure 1. Possible metabolic pathways of *d*-limonene



From Kodama *et al.* (1976)
M-I, *p*-Mentha-1,8-dien-10-ol; M-II, *p*-menth-1-ene-8,9-diol; M-IV, perillic acid-8,9-diol; M-V, *p*-mentha-1,8-dien-10-yl-β-D-glucopyranosiduronic acid; M-VI, 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid; M-VII, 2-hydroxy-*p*-menth-8-en-7-oic acid; M-VIII, perillylglycine; M-IX, perillyl-β-D-glucopyranosiduronic acid; M-X, *p*-mentha-1,8-dien-6-ol; M-XI, *p*-menth-1-ene-6,8,9-triol

In the phase-I clinical trial of orally administered *d*-limonene described in section 4.1.1, toxicity was limited to gastrointestinal symptoms (irritation, nausea, diarrhoea) and was dose-related over the range 6.5–12 g/m^2 body surface area per day (Vigushin *et al.*, 1998).

4.2.2 *Experimental systems*

The LD_{50} values for *d*-limonene in male and female mice were reported to be 5.6 and 6.6 (oral), 1.3 and 1.3 (intraperitoneal) and > 42 and > 42 (subcutaneous) g/kg bw, respectively; those in male and female rats were reported to be 4.4 and 5.1 (oral), 3.6 and 4.5 (intraperitoneal), > 20 and > 20 (subcutaneous) and 0.12 and 0.11 (intravenous) g/kg bw, respectively (Tsuji *et al.*, 1975a). The acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits were reported to exceed 5 g/kg bw (Opdyke, 1975).

After daily administration of *d*-limonene at 277–2770 mg/kg bw to male and female Sprague-Dawley rats for one month, the highest dose was found to have caused a slight decrease in body weight and food consumption. On histological examination (staining with hematoxylin and eosin), granular casts were observed in the kidney of males, but no significant change was found in the other organs (Tsuji *et al.*, 1975a).

A dose-related increase in relative liver and kidney weights was observed in young adult male Fischer 344 rats given 75, 150 or 300 mg/kg bw *d*-limonene daily by gavage on five days per week and killed on study days 6 or 27. With Mallory-Heidenhain staining, a dose-related formation of hyaline droplets was observed histologically in the kidneys. Hyaline droplet nephropathy was associated with increased concentrations of α_{2u}-globulin in renal cortical homogenates separated by two-dimensional gel electrophoresis. The concentrations of other renal proteins were not increased by *d*-limonene treatment. Alterations considered to be sequelae of the hyaline droplet response, including granular casts in the outer zone of the medulla and multiple cortical changes collectively classified as chronic nephrosis, were observed in the kidneys of all rats killed on day 27 (Kanerva *et al.*, 1987).

Oral administration of 75 or 150 mg *d*-limonene to male Fischer 344/N rats on five days per week for two years was associated with dose-related alterations to the kidney, such as increased incidences of mineralization and epithelial hyperplasia and increased severity of chronic progressive nephropathy. In the same study, no signs of toxicity, including renal hyaline droplet formation, were observed in female Fischer 344/N rats dosed at 300 or 600 mg/kg bw (National Toxicology Program, 1990).

Male and female $B6C3F_1$ mice were treated orally on five days a week for two years with doses of 250 or 500 and 500 or 1000 mg/kg bw, respectively. The mean body weights of females at the high dose were 5–15% lower than those of controls after week 28, but no compound-related toxicity was observed in animals of either sex (National Toxicology Progam, 1990).

In beagle dogs, oral doses of more than 340 mg/kg bw (bitches) and 1000 mg/kg bw (dogs) per day for six months resulted in protein casts in the renal tubules. Daily doses of more than 1000 mg/kg bw (bitches) and 3024 mg/kg bw (dogs) resulted in slight weight loss due to frequent vomiting in some animals (Tsuji *et al.*, 1975b).

In another study in adult beagle dogs, *d*-limonene at 100 or 1000 mg/kg bw (maximal tolerated dose for emesis) per day by gavage twice daily for six months increased kidney weights but induced no histopathological changes, hyaline droplet accumulation or nephropathy (Webb *et al.*, 1990).

d-Limonene given orally for four days at 1650 mg/kg bw per day caused no renal toxicity in male NCI Black Reiter rats, which do not synthesize the α_{2u}-globulin that is normally present in hyaline droplets found in male Fischer 344 rats with *d*-limonene-induced nephrotoxicity (Dietrich & Swenberg, 1991a).

The ability of *d*-limonene to cause hyaline droplet nephropathy was evaluated in C57BL/6-derived transgenic mice engineered to express α_{2u}-globulin. These mice excreted approximately 30% less α_{2u}-globulin than male rats. α_{2u}-Globulin was detected in the kidney by immunoblotting; after *d*-limonene treatment at 150 mg/kg bw for three days, the concentration of α_{2u}-globulin was increased threefold relative to untreated controls. Spontaneous hyaline droplet formation was not seen in control transgenic mice, but small droplets were observed after *d*-limonene treatment (Lehman-McKeeman & Caudill, 1994).

d-Limonene at 150 mg/kg bw increased renal-cell proliferation in male Fischer 344 rats, particularly in the P_2 segment of the renal proximal tubular epithelium, after 4 or 31 weeks of exposure. Cell proliferation, determined by bromodeoxyuridine labelling, was increased approximately fivefold over that in control rats. No increase in renal-cell proliferation was observed in male NBR rats treated similarly in the same experiment (Dietrich & Swenberg, 1991b).

d-Limonene increased renal-cell proliferation in response to hyaline droplet exacerbation in Fischer 344 rats dosed orally for 91 days at 0, 5, 30, 75 or 150 mg/kg bw. No exacerbation of hyaline droplets was noted at the lowest dose, and there was no increase in proliferating cell nuclear antigen-labelled renal proximal tubular cells. At doses of 30 mg/kg bw *d*-limonene and higher, both hyaline droplet formation and the percentage of labelled cells were increased. At the highest dose, the percentage of antigen-labelled cells was increased by about six times over that in controls, and the cells were localized to the P_2 segment of the proximal tubule (Lehman-McKeeman, 1995).

mRNA for α_{2u}-globulin was markedly reduced or was undectable in the livers of male rats [strain not specified] fed diets containing the peroxisome proliferator and lipid-lowering agent ciprofibrate (0.025% wt/wt) for seven weeks. This finding was confirmed by immunoblot analysis with antibodies against α_{2u}-globulin, although immunohistochemical staining and in-situ hybridization showed the presence of a few cells that contained α_{2u}-globulin protein and its m-RNA. The α_{2u}-globulin m-RNA reappeared in the liver two weeks after cessation of ciprofibrate treatment. Feeding of ciprofibrate for two weeks, followed by simultaneous feeding of ciprofibrate and *d*-limonene (150 mg/kg bw per day, six days a week), showed that ciprofibrate prevented α_{2u}-globulin accumulation and the nephrotoxicity associated with binding of *d*-limonene to this protein (Alvares *et al.*, 1996).

4.3 Reproductive and developmental effects

4.3.1 *Humans*

No data were available to the Working Group.

4.3.2 *Experimental systems*

Studies in mice, rats and rabbits exposed by oral administration during organogenesis showed impaired weight gains in the dams and a transient reduction in the growth of the offspring. Anomalies of the ribs were observed in offspring of mice and rats (IARC, 1993).

It was reported in an abstract that *d*-limonene was not teratogenic to frog embryos (*Xenopus laevis*) although a teratogenic effect was reported at the lowest concentration (0.00114 mg/L) (Holck *et al*., 1991).

4.4 Genetic and related effects

4.4.1 *Humans*

No data were available to the Working Group.

4.4.2 *Experimental systems* (see Table 1 for references)

d-Limonene was not mutagenic to *Salmonella typhimurium*. In single studies, it did not induce sister chromatid exchange, chromosomal aberrations, trifluorothymidine resistance or transformation of rodent cells *in vitro*. *d*-Limonene gave negative results in the mammalian spot test even at toxic doses.

d-Limonene has been found to inhibit gap-junctional intercellular communication in mouse primary keratinocytes and derived cell lines.

The metabolite *d*-limonene-1,2-oxide gave negative results in the SOS chromotest. It was not mutagenic to *S. typhimurium* and did not induce unscheduled DNA synthesis in rat hepatocytes *in vitro*. Essential oils containing *d*-limonene did not induce differential toxicity in *Bacillus subtilis*, nor did they induce reverse mutation in *S. typhimurium*.

d-Limonene did not inhibit the transformation of rat tracheal epithelial cells by benzo[*a*]pyrene (Steele *et al*., 1990).

4.5 Mechanistic considerations

4.5.1 *Renal tumours in male rats*

The criteria for establishing that an agent causes renal tumours in male rats through a response associated with α_{2u}-globulin (Capen *et al*., 1999) are as follows:

- lack of genotoxic activity (the agent and/or a metabolite) on the basis of an overall evaluation of results obtained *in vitro* and *in vivo*;
- nephropathy and renal tumorigenicity seen only in male rats;
- induction in shorter studies of the characteristic sequence of histopathological changes, of which protein droplet accumulation is obligatory;
- identification of the protein that accumulates in tubular cells as α_{2u}-globulin;
- reversible binding of the chemical or metabolite to α_{2u}-globulin;
- induction of sustained increased cell proliferation in the renal cortex; and
- similarities in dose–response relationship of the tumour outcome with those for histopathological end-points (protein droplets, α_{2u}-globulin accumulation, cell proliferation).

Table 1. Genetic and related effects of *d*-limonene and its metabolites

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
***d*-Limonene**				
Salmonella typhimurium TA100, TA98, TA1535, TA1537, TA1538, reverse mutation	–	–	2720 µg/plate	Watabe *et al.* (1981)
Salmonella typhimurium TA100, TA98, TA1535, TA1537, reverse mutation	–	–	3333 µg/plate	Haworth *et al.* (1983)
Gene mutation, mouse lymphoma L5178Y cells, *tk* locus *in vitro*	–	–	60	National Toxicology Program (1990)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	162	National Toxicology Program (1990)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	–	500	National Toxicology Program (1990)
Inhibition of intercellular communication mouse 3PC cells *in vitro*	+	NT	136	Jansen & Jongen (1996)
Cell transformation, Syrian hamster embryo cells	–	NT	50	Oshiro *et al.* (1998)
Mammalian spot test, mouse *in vivo*	–		215 ip × 3	Fahrig (1982)
***d*-Limonene-1,2-oxide**				
Escherichia coli PQ37, induction of SOS repair	–	–	500	von der Hude *et al.* (1990a)
Salmonella typhimurium TA100, TA1535, reverse mutation	–	–	500 µg/plate	von der Hude *et al.* (1990a)
Unscheduled DNA synthesis, primary rat hepatocytes *in vitro*	–	NT	15	von der Hude *et al.* (1990b)

Table 1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Essential oils containing *d*-limonene				
Bacillus subtilis rec strains, differential toxicity	–	NT	30 µL/plate	Zani *et al.* (1991)
Salmonella typhimurium TA100, TA98, TA1535, TA1537 reverse mutation	–	–	2.5 µL/plate	Zani *et al.* (1991)

[a] +, positive; –, negative; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; unless otherwise stated, in-vitro test, µg/mL; in-vivo test, mg/kg bw per day; ip, intraperitoneal

These criteria are met by *d*-limonene, which produces hyaline droplet nephropathy and causes renal tubular tumours in male rats through an α_{2u}-globulin-associated response.

d-Limonene causes a renal syndrome that occurs exclusively in male rats. Male rats are unique in that they exhibit a background of spontaneous protein droplets in the proximal tubule, particularly within the cells of the P_2 segment. *d*-Limonene exacerbates the formation of these droplets, and it was shown by two-dimensional gel electrophoresis that the only protein accumulating was α_{2u}-globulin (Alden *et al.*, 1984; Kanerva *et al.*, 1987).

α_{2u}-Globulin is synthesized in the liver, secreted into the general circulation and reabsorbed by renal proximal tubule cells. Hepatic synthesis of α_{2u}-globulin occurs only in adult male rats (Roy *et al.*, 1966). The critical role of α_{2u}-globulin in the renal effects of *d*-limonene is demonstrated by the absence of histopathological changes in female rats and in species that do not synthesize α_{2u}-globulin. Thus, *d*-limonene shows no renal toxicity in female rats, in male NBR rats or in other species studied, including male and female mice and dogs (Chatterjee *et al.*, 1989; Dietrich & Swenberg, 1991a). *d*-Limonene also has no renal toxicity in ciprofibrate-treated male rats in which synthesis of α_{2u}-globulin is repressed (Alvares *et al*., 1996). Whereas mice do not develop hyaline droplet nephropathy, transgenic mice that synthesize α_{2u}-globulin develop these lesions (Lehman-McKeeman & Caudill, 1994).

The mechanism by which *d*-limonene causes α_{2u}-globulin accumulation in the male rat kidney has been elucidated. The prerequisite step in the development of the nephropathy is the binding to α_{2u}-globulin of an agent, which in the case of *d*-limonene is the 1,2-epoxide (Lehman-McKeeman *et al.*, 1989). This binding is specific for α_{2u}-globulin and reversible, with a binding affinity (K_d) of approximately 5.6×10^{-7} mol/L (Borghoff *et al.*, 1991; Lehman-McKeeman & Caudill, 1992a). Binding of this ligand to α_{2u}-globulin reduces the rate of its lysosomal degradation relative to that of native protein, thereby causing it to accumulate. Lysosomal cathepsin activity towards other protein substrates is not altered (Lehman-McKeeman *et al.*, 1990).

Whereas accumulation of α_{2u}-globulin can be observed after a single oral dose of *d*-limonene, continued treatment results in additional histological changes in the kidney. Phagolysosomes become enlarged, engorged with protein and show polyangular crystalloid inclusions. After three to four weeks of dosing, progressive renal injury, characterized by single-cell degeneration and necrosis in the P_2 segment of the renal proximal tubule, is noted. Dead cells are sloughed into the lumen of the nephron, contributing to the development of granular casts at the cortico-medullary junction (Swenberg & Lehman-McKeeman, 1999). Renal functional perturbations, including reduced uptake of organic anions, cations and amino acids and mild proteinuria resulting from a large increase in the amount of α_{2u}-globulin excreted in urine, are observed. These functional changes occur only in male rats and only at doses that exacerbate the protein droplet formation (Swenberg & Lehman-McKeeman, 1999). In response to the cell death and functional changes, there is a compensatory increase in cell proliferation in the kidney, most notably in the P_2 segment of the proximal tubules, the site of protein accumulation

(Dietrich & Swenberg, 1991b; Swenberg & Lehman-McKeeman, 1999). With continued treatment, the cell proliferation persists, but it does not restore renal function. The increase in cell proliferation is linked to the development of renal tubular tumours (Dietrich & Swenberg, 1991b). α_{2u}-Globulin nephropathy and renal-cell proliferation occur at doses consistent with those that produce renal tubular tumours.

The link between α_{2u}-globulin nephropathy and renal-cell proliferation was established for *d*-limonene in comparative studies of male NBR and Fischer 344 rats. Cell proliferation in renal tubules was increased by *d*-limonene in Fischer 344 rats after 4 or 31 weeks of exposure to *d*-limonene but not in NBR rats which do not develop α_{2u}-globulin nephropathy. Thus, the increase in cell proliferation was shown to be totally dependent on the presence of α_{2u}-globulin. Furthermore, when evaluated in an initiation–promotion study with *N*-nitrosoethyl-*N*-hydroxyethylamine, *d*-limonene treatment increased the incidence of renal adenomas in Fischer 344 rats, whereas no renal tumours occurred in NBR rats (Dietrich & Swenberg, 1991b).

The available data indicate that renal tubular tumours in male rats develop by a secondary, non-DNA reactive mechanism. Histopathological, biochemical and cell proliferation investigations provide compelling evidence that *d*-limonene produces a syndrome that begins acutely as accumulation of α_{2u}-globulin but represents a continuum of changes that progress ultimately to renal tubular tumours (Swenberg *et al.*, 1989; Flamm & Lehman-McKeeman, 1991; Hard *et al.*, 1993; Hard & Whysner, 1994; Swenberg & Lehman-McKeeman, 1999).

Relevance to humans

The requisite step in the development of α_{2u}-globulin nephropathy is binding of *d*-limonene, and particularly the 1,2-epoxide, to α_{2u}-globulin. α_{2u}-Globulin is a member of a superfamily of proteins that bind and transport a variety of ligands. Many of these proteins are synthesized in mammalian species, including humans. Therefore, the question of whether a similar mechanism occurs in humans can be addressed by determining whether these structurally homologous proteins function in humans in a manner analogous to α_{2u}-globulin. This question has been answered both qualitatively and quantitatively.

The protein content of human urine is very different from that of rat urine, as humans excrete very little protein (about 1% of the concentration found in urine of male rats). Human urinary protein is also predominantly a species of high molecular mass, and there is no protein in human plasma or urine identical to α_{2u}-globulin (Olson *et al.*, 1990). No α_{2u}-globulin-like protein has been detected in human kidney tissue (Borghoff & Lagarde, 1993). Saturable binding of *d*-limonene-1,2-epoxide to α_{2u}-globulin can be shown *in vitro*, but other superfamily proteins, particularly those synthesized by humans, do not bind this agent (Lehman-McKeeman & Caudill, 1992b).

The unique specificity of the syndrome of renal toxicity in male rats due to α_{2u}-globulin is demonstrated by the lack of toxicity and of renal tumours in mice. Mice synthesize mouse urinary protein, which shares nearly 90% sequence identity to α_{2u}-globulin; however, *d*-limonene-1,2-epoxide does not bind to the mouse protein and it does

not produce a similar syndrome in mice. Additionally, the lack of a response in female rats, which synthesize many other proteins of the superfamily, demonstrates that these proteins are unlikely to contribute to renal toxicity.

The X-ray crystal structure of α_{2u}-globulin has been derived and indicates that although α_{2u}-globulin may share amino acid sequence with many other proteins it has a unique ligand-binding pocket. Other superfamily proteins are characterized by flattened, elongated binding pockets, whereas the ligand-binding site in α_{2u}-globulin is distinguished by a spherical, non-restrictive shape. The elongated, flattened binding pockets in mouse urinary protein and in superfamily proteins synthesized by humans preclude the binding of *d*-limonene-1,2-epoxide to these proteins (Lehman-McKeeman, 1997).

From a quantitative perspective, adult male rat kidneys reabsorb about 35 mg α_{2u}-globulin per day. Female rats synthesize less than 1% of the amount of α_{2u}-globulin reabsorbed by male rats, but no α_{2u}-globulin is detected in female rat kidney and female rats do not develop nephropathy. The most abundant α_{2u}-globulin superfamily protein in human kidney and plasma is α_1-acid glycoprotein, and this protein does not bind to agents that induce α_{2u}-globulin nephropathy in rats.

Taken together, there is no evidence that any human protein can contribute to a renal syndrome similar to α_{2u}-globulin nephropathy, and thus no evidence that *d*-limonene is carcinogenic in humans by a mechanism similar to α_{2u}-globulin nephropathy.

The induction of renal-cell tumours in male rats by agents that act through an α_{2u}-globulin-associated response is not predictive of carcinogenic hazard to humans (Capen *et al.*, 1999). This conclusion is based on extensive evidence that the presence of α_{2u}-globulin is an absolute requirement for the carcinogenic activity and that neither α_{2u}-globulin nor any protein that can function like α_{2u}-globulin is synthesized by humans (Swenberg & Lehman-McKeeman, 1999).

d-Limonene has no carcinogenic activity at any other site in male rats. Consequently, all of the mechanistic data support the conclusion that the renal tumours in male rats produced by *d*-limonene are not relevant to humans.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

d-Limonene is a terpene which occurs naturally in citrus and a variety of other plants. Exposure occurs from its presence in foods and its use as a solvent. It is being evaluated in clinical trials for use as a cancer chemotherapeutic agent.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

d-Limonene was tested for carcinogenicity by oral gavage in mice and rats and in several two-stage experiments with multi-organ carcinogens. It significantly increased the incidence of renal tubular tumours (adenomas and carcinomas) and induced atypical renal tubular hyperplasia in male rats, which normally synthesize α_{2u}-globulin in the liver, but not in female rats or in mice of either sex. It consistently enhanced the incidences of renal tubular tumours and atypical renal tubular hyperplasia initiated by carcinogens in two-stage carcinogenesis assays in male rats of a strain conventionally used in bioassays, but not in a strain that lacks hepatic synthesis of α_{2u}-globulin.

d-Limonene was tested as a cancer-preventive agent in other experimental models with known carcinogens. It inhibited lung carcinogenesis in mice, preneoplastic stages of colon carcinogenesis in rats and pancreatic carcinogenesis in hamsters.

5.4 Other relevant data

d-Limonene is metabolized in humans and experimental animals to a variety of metabolites, including perillic acid and *d*-limonene-1,2-diol. *d*-Limonene causes a male rat-specific nephrotoxicity resulting from accumulation of the male rat-specific protein α_{2u}-globulin. *d*-Limonene-1,2-epoxide binds reversibly to α_{2u}-globulin. *d*-Limonene causes sustained cell proliferation in renal proximal tubular cells, and the dose–response relationships for tumour outcome, enhanced cell proliferation and other histopathological end-points typical of α_{2u}-globulin nephropathy are similar. Female rats, male rats of strains that do not express this protein and other species are not susceptible to the nephrotoxic action of *d*-limonene.

Developmental toxicity in the form of delayed prenatal growth has been observed in mice, rats and rabbits exposed to *d*-limonene during gestation. Skeletal anomalies have also been observed in the fetuses of exposed mice and rabbits.

The few available data indicate that *d*-limonene and its 1,2-epoxide metabolite are not genotoxic.

5.5 Evaluation

There is *inadequate evidence* in humans for the carcinogenicity of *d*-limonene.

There is *sufficient evidence* in experimental animals for the carcinogenicity of *d*-limonene.

Overall evaluation

In making its overall evaluation of the carcinogenicity to humans of *d*-limonene, the Working Group concluded that *d*-limonene produces renal tubular tumours in male rats by a non-DNA-reactive mechanism, through an α_{2u}-globulin-associated response. Therefore, the mechanism by which *d*-limonene increases the incidence of renal tubular tumours in male rats is not relevant to humans.

d-Limonene is *not classifiable as to its carcinogenicity to humans (Group 3).*

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