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Review

FEMA expert panel review of *p*-mentha-1,8-dien-7-al genotoxicity testing results

Samuel M. Cohen [a], Shoji Fukushima [b], Nigel J. Gooderham [c], F. Peter Guengerich [d], Stephen S. Hecht [e], Ivonne M.C.M. Rietjens [f], Robert L. Smith [g], Maria Bastaki [h], Christie L. Harman [h], Margaret M. McGowen [h], Sean V. Taylor [h, *]

[a] Dept. of Pathology and Microbiology, University of Nebraska Medical Center, 983135 Nebraska Medical Center, Omaha, NE 68198-3135, USA
[b] Japan Bioassay Research Center, 2445 Hirasawa, Hadano, Kanagawa 257-0015, Japan
[c] Dept. of Surgery and Cancer, Imperial College of London, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom
[d] Dept. of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146, USA
[e] Wallin Professor of Cancer Prevention, Masonic Cancer Center and Dept. of Laboratory Medicine and Pathology, University of Minnesota, Cancer and Cardiovascular Research Building, 2231 6th St. SE, Minneapolis, MN 55455, USA
[f] Division of Toxicology, Wageningen University, Stippeneng 4, 6708 WE, Wageningen, The Netherlands
[g] Molecular Toxicology, Imperial College School of Medicine, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom
[h] Flavor and Extract Manufacturers Association, 1101 17th Street, NW Suite 700, Washington, DC 20036, USA

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ABSTRACT

p-Mentha-1,8-dien-7-al is a naturally occurring cyclic alpha,beta-unsaturated aldehyde that is used as a flavoring substance throughout the world. Due to the chemical structure and the potential DNA reactivity of the alpha,beta-unsaturated carbonyl moiety, a battery of genotoxicity assays was requested by the European Food Safety Authority. Previous genotoxicity studies on the substance gave mixed results, but both positive and negative results were hampered by not always being performed to any standard guideline. The new test battery data indicated some evidence of mutagenicity *in vitro*, but an *in vivo* comet/micronucleus combination assay performed in rats was concluded by the study directors to not result in any biologically relevant positive responses. However, EFSA concluded that the *in vivo* assay gave evidence that *p*-mentha-1,8-dien-7-al was of potential genotoxic concern. The Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) has reviewed the newly available data and considered its interpretation relative to standard guidelines such as that established by the Organization for Economic Cooperation and Development, and has concluded that the results in the comet/micronucleus combination assay are consistent with the interpretation by the study directors; namely, that *p*-mentha-1,8-dien-7-al does not appear to have any *in vivo* genotoxic potential.

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Abbreviations: bw, body weight; CA, chromosomal aberration; EMS, ethylmethanesulfonate; EFSA, the European Food Safety Authority; FEMA, the Flavor and Extract Manufacturers Association; GLP, good laboratory practice; *i.p.*, intraperitoneal; JECFA, Joint FAO/WHO Expert Committee on Food Additives; M, male; MN, micronucleus; MNBN cells, micronucleated binucleate cells; MSDI, maximized survey-derived intake; MTD, maximum tolerated dose; NA, data not available; NAS, National Academy of Science; NOAEL, no-observed-adverse-effect level; NOEL, no-observed-effect level; NTP, National Toxicology Program; OECD, Organization for Economic Cooperation and Development; ppm, parts per million; S-9, metabolic activation system; SCE, sister chromatid exchange.

* Corresponding author.
E-mail addresses: scohen@unmc.edu (S.M. Cohen), 2940-shoji@kxb.biglobe.ne.jp (S. Fukushima), n.gooderham@imperial.ac.uk (N.J. Gooderham), f.guengerich@vanderbilt.edu (F.P. Guengerich), hecht002@umn.edu (S.S. Hecht), ivonne.rietjens@wur.nl (I.M.C.M. Rietjens), rrlsmith@btinternet.com (R.L. Smith), mbastaki@vertosolutions.net (M. Bastaki), charman@vertosolutions.net (C.L. Harman), mmcgowen@vertosolutions.net (M.M. McGowen), staylor@vertosolutions.net (S.V. Taylor).

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1. Introduction

A number of genotoxicity tests have been developed and validated over time that can provide relevant information regarding the potential of a substance to damage DNA. Yet, no single test is sufficient in isolation to provide definitive answers to the question of whether a substance is genotoxic or not. Hence, scientific bodies and regulators alike have recognized the value of a genotoxicity testing battery that includes a minimum set of assays, whether *in vitro* or *in vivo*, each with the power to reveal different modes of genotoxic potential.

Considering the strengths and specific parameters that each type of genotoxicity assay offers, most testing batteries that are now used to address the genotoxic potential of chemicals, including a variety of food ingredients, include a combination adequate to thoroughly assess genotoxic potential. Regulatory guidelines in the US (Redbook) and Europe (European Food Safety Authority (EFSA) Genotoxicity Testing Guidelines) have been developed to specify the minimum set of complementary tests to obtain a complete picture of potential genotoxicity for food substances, covering three genotoxicity endpoints, namely mutagenicity, structural (clastogenicity), and numerical (aneugenicity) chromosomal aberrations (FDA, 2007; EFSA, 2011). Similar genotoxicity testing batteries have been developed for pharmaceutical substances (ICH, 2011).

For most food ingredients, a basic battery typically includes an *in vitro* test of mutagenic potential and an *in vitro* genotoxicity test that addresses the potential for clastogenic and/or aneugenic effects. Specifically, studies to investigate gene (point) mutations include a) a bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* [Organization for Economic Cooperation and Development Testing Guideline (OECD TG) 471] and b) an *in vitro* mammalian cell gene mutation test (OECD TG 476); and studies to investigate chromosome aberrations include a) an *in vitro* mammalian chromosomal aberration test (OECD 473) and b) an *in vitro* mammalian cell micronucleus test (OECD TG 487). Of these, the bacterial reverse mutation test (OECD TG 471) and the *in vitro* mammalian cell micronucleus test (OECD TG 487) are the most common *in vitro* set required for regulatory testing. This set may be modified or abandoned if there are reasons to consider it insufficient, such as evidence that specific metabolic pathways for a certain substance occurring in humans are absent in the *in vitro* test system, or that the *in vitro* test system is inappropriate on grounds of physicochemical properties of the substance or of its mode of action.

Following up *in vitro* testing with *in vivo* assays is generally neither needed nor encouraged if results of *in vitro* tests are clearly negative. However, where positive or unclear results are collected from one or more *in vitro* test systems, they may be followed with an appropriate *in vivo* assay that further assesses the biological relevance of the *in vitro* findings *in vivo*. In light of equivocal and especially positive responses in the *in vitro* tests, selected *in vivo* tests are recommended based on the type of genotoxic endpoint that needs further resolution, specifically, a) a mammalian erythrocyte micronucleus test (OECD TG 474) to follow up an equivocal or positive *in vitro* micronucleus test; this may be substituted by a bone marrow chromosomal aberrations test (OECD TG 475); b) transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488) to follow up equivocal or positive *in vitro* mutagenicity assays; and c) an *in vivo* comet assay (OECD TG 489); this is considered a useful "indicator" test to follow up equivocal or positive *in vitro* mutagenicity or clastogenicity (but not aneugenicity) results.

Each genotoxicity assay has been thoroughly evaluated by groups of experts who have reached consensus on the methodological details, the evaluation and acceptance criteria of test validity and a set of criteria for the interpretation of results, including statistical analysis. Collectively the consensus conclusions on the utility, strengths, limitations, and performance of the assay are made public in the form of OECD Guidelines, which are periodically updated and refined as new information or experience with the conduct of the assays becomes available. The intent of the Guidelines is to provide a set of minimum quality criteria for each assay so that when performed by any investigator anywhere in the world they can provide results that are directly comparable to those of similarly run assays. More importantly, the Guidelines intend to lay down specific criteria for the interpretation of results. One readily appreciates the challenge of drawing conclusions about the biological properties of a chemical substance—genotoxicity in this case—when considering the complexity of biological systems. Results that are clearly negative or clearly positive present no challenge. Biological deviations, however, can result in artefacts in the test system and this is where the utmost scientific rigor and a solid understanding of the system and the assay performed are paramount to reaching scientifically justifiable conclusions. The possibility of randomness in biological events can generally be controlled to an extent by the application of statistical procedures to assist in interpreting variations by providing a gauge of the likelihood that they are truly random. The OECD Guidelines recognise this and prescribe appropriate statistical applications. Statistical analysis appropriate for the type of data is applied to determine whether a variation from what is naturally variable is large enough (magnitude of change) to be considered non-random and an effect of the test conditions, such as the presence of the test substance. This is determined both against the variation within the specific assay by comparing the treatment to the internal or concurrent control, but also against the variation that the system has

presented historically from all previous occasions when it was performed, indicated as a range of "normal" outcomes, or the historical vehicle control range. In every OECD Guideline these elements are present without exception, revealing the consensus understanding by experts in all areas of genotoxicity testing of the challenges in interpreting randomness and biological variability within experiments.

Despite the consensus to explicitly establish criteria for the interpretation of assay results, unintentional violations can still occur. Within this commentary, the FEMA Expert Panel evaluates the available genotoxicity data for *p*-mentha-1,8-dien-7-al, which was also recently the focus of an opinion published by EFSA (EFSA, 2015). In that opinion, EFSA concluded that the substance, based on the available data, demonstrated genotoxic potential. In the review provided here, the FEMA Expert Panel has concluded that *p*-mentha-1,8-dien-7-al does not raise a concern with respect to genotoxicity. The Panel's review of the data, as well as a discussion of the differing conclusions, is provided.

2. The genotoxicity testing profile of *p*-mentha-1,8-dien-7-al

p-Mentha-1,8-dien-7-al (CAS No. 2111-75-3), also known as perillaldehyde, is a flavoring substance that is approved for use in the USA (FEMA No. 3557) and is currently considered of no safety concern under current conditions of use by the Joint FAO/WHO Expert Committee on Food Additives. It was considered by EFSA to be a "representative" substance of a chemical group of 10 alicyclic aldehydes, alcohols and their esters with the alpha, beta (α,β)-unsaturation in the ring or side-chain[1] (EFSA, 2008). On that basis, EFSA requested data for *p*-mentha-1,8-dien-7-al according to their published genotoxicity testing guidance (EFSA, 2011).

EFSA identified the feature of α,β-unsaturation as a structural alert for genotoxicity since it could potentially give rise to reactivity with cellular nucleophiles, including DNA (EFSA, 2008). EFSA created a list of more than 300 flavorings based on the α,β-unsaturation structural feature or the ability of flavorings to be converted into substances that would contain this structural feature. This list is referred to as Flavoring Group Evaluation 19 (FGE.19), which covers all α,β-unsaturated aldehydes or ketones and their precursors. The FGE.19 list was further divided into subgroups based on additional structural features, and a group of 10 flavorings, including *p*-mentha-1,8-dien-7-al, was allocated to FGE.19 chemical subgroup 2.2. For subgroup 2.2, EFSA determined that there was a need for additional information before conclusions on their genotoxicity could be reached.

2.1. Genotoxicity testing (prior to 2010)

2.1.1. In vitro

p-Mentha-1,8-dien-7-al was tested in several *in vitro* genotoxicity assays that provided inconsistent information about its genotoxic potential (Fujita et al., 1994; Hayashi et al., 1988; Ishidate et al., 1984; Kuroda et al., 1984; Sasaki et al., 1990; Suzuki and Suzuki, 1994; Tayama et al., 1990; Yoo, 1986) (Table 1).

In assays that explored the mutagenic potential, *p*-mentha-1,8-dien-7-al was negative in bacterial reverse mutation assays (Ames assays) in *Salmonella typhimurium* strains TA92, TA94, TA98, TA100, TA1535, and TA1537 at concentrations up to 1000 μg/plate, with and without metabolic activation (Ishidate et al., 1984), or with *S. typhimurium* strains TA97 and TA102 at concentrations up to 100 μg/plate, with and without metabolic activation (Fujita et al., 1994) and with *Escherichia coli* WP2 at concentrations from 50 to 400 μg/plate in the absence of metabolic activation (inclusion of a metabolic activation system was not examined) (Yoo, 1986). While there were no indications of positive results, the concentrations used in these studies are not consistent with current OECD guidelines (OECD, 1997).

In the *rec* assay with *Bacillus subtilis* strains M45 (*rec*$^-$) and H17 (*rec*$^+$), one study reported negative results at concentrations between 0.16 and 0.63 μl/plate and positive results at higher concentrations (1.25–2.5 μl/plate) (Kuroda et al., 1984). In the *Rec* assay of Yoo (1986), a weak positive response was seen at the highest concentration of 2.5 μl/plate (using a specific density of ~0.965 g/ml, this is equivalent to ~2400 μg/plate) (Yoo, 1986). However, there was no growth inhibition in the H17 *rec*$^+$ strain (inhibition zone = 0 mm), and the zone of growth inhibition in M45 *rec*$^-$ was low (12 mm). It should be noted that the bacterial DNA damage (*Rec*) assays are not mutation assays. Therefore, this information can only be considered as auxiliary.

Negative results were reported in the SOS Chromotest, which uses genetically engineered *E. coli*, with a "maximum induction factor" (I_{max}) of 1.0 compared to the minimum I_{max} threshold of significance of 1.5 (Eder et al., 1993). The SOS chromotest is an indicator of DNA damage and strictly not a mutation test. There is no information on what concentrations were tested, how many replicates etc., and therefore these data are inadequate for assessment.

Inconsistent results have been reported in standard cytogenetic assays, such as Sister Chromatid Exchange (SCE) and chromosomal aberration (CA) assays with and without metabolic activation. One SCE assay, conducted in Chinese hamster ovary cells (CHO-K1), yielded positive results for genotoxicity at concentrations of 50–100 μg/ml without S-9, and 50–300 μg/ml with S-9, with cytotoxicity reported at 150 μg/ml in the absence of S-9 (Tayama et al., 1990), while another SCE assay, also in CHO-K1 cells, yielded negative results at 10 μg/ml with cytotoxicity reported at concentrations ≥12.5 μg/ml (Sasaki et al., 1990). Similarly, one CA study reported positive results in the Chinese hamster fibroblast cell line (CHL) at 40 and 50 μg/ml (with 20% and 39% incidence, respectively) without metabolic activation (Ishidate et al., 1984). However, in the Ishidate et al. (1984) study, there was no concurrent measure of cytotoxicity. Instead, the test concentrations were selected from a preliminary experiment in which only cell density was measured as indicator of cytotoxicity, a rather crude and subjective measure. In addition, only single cultures were treated with each of three concentrations, and therefore only 100 cells from each concentration and time point were scored for CA, which is significantly less than OECD guidelines mandate. In another study using 50–300 μg/ml, CA increases were reported only at the top concentrations without and with S-9 (150 μg/ml and 300 μg/ml, respectively) which were also associated with cytotoxicity (Tayama et al., 1990). The SCE assays only provide limited information since the mechanism of SCE induction and its relevance for mutation and cancer are not well understood.

In a mutation assay conducted in CHO cells using ouabain resistance as a marker, *p*-mentha-1,8-dien-7-al was tested at a single concentration of 10 μg/ml, which reduced survival to 63.5% of controls (Sasaki et al., 1990). An increase in mutant frequency was reported (0.7 mutants per 10^6 cells) versus zero mutations in controls. This level of mutation is very low and the result would probably be considered negative. However, the statistical significance, dose-dependence, or performance of the assay or the test substance relative to historical control data were not determined,

[1] *p*-Mentha-1,8-dien-7-ol [FL-no: 02.060]; Myrtenol [FL-no: 02.091]; Myrtenal [FL-no: 05.106]; *p*-Mentha-1,8-dien-7-al [FL-no: 05.117]; 2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121]; Myrtenyl formate [FL-no: 09.272]; *p*-Mentha-1,8-dien-7-yl acetate [FL-no: 09.278]; Myrtenyl acetate [FL-no: 09.302]; Myrtenyl-2-methylbutyrate [FL-no: 09.899]; Myrtenyl-3-methylbutyrate [FL-no: 09.900].

so the significance of these results is unclear.

In two mutagenicity studies in human fetal cells (Rsa), positive results were reported at very low concentrations, based on ouabain resistance as a mutagenicity indicator. In one study, *p*-mentha-1,8-dien-7-al was negative at 10 ng/ml and positive at 15 and 20 ng/ml, where it induced significant (>8-fold, and >15-fold, respectively) increases in ouabain resistance. It was also negative at a cytotoxic concentration of 25 ng/ml (Suzuki et al., 1990). In another study (Suzuki and Suzuki, 1994), *p*-mentha-1,8-dien-7-al was mutagenic at >10 ng/ml as determined by ouabain resistance, and at 2–200 ng/ml as determined at *K-ras* codons, with clear indications of cytotoxicity at 20 ng/ml and higher. The ouabain resistance locus is not currently used as a marker for mutagenicity in a regulatory testing context. In addition, human fetal (Rsa) cells are not routinely used for genotoxicity testing.

2.1.2. *In vivo*

In an *in vivo* micronucleus assay, *p*-mentha-1,8-dien-7-al was administered to eight-week-old male ddY mice via a single intraperitoneal injection at doses of 75, 150, 300, or 600 mg/kg bw (6 mice/group) (Table 2). The mice were killed after 24 h and femoral bone marrow cells were collected and processed for analysis. There was no evidence of micronucleus induction at any dose level, based on scoring of 1000 polychromatic erythrocytes per mouse (Hayashi et al., 1988). It is of interest to note that this *in vivo* bone marrow MN test of Hayashi et al. (1988) does not comply with current guidelines. Specifically, after a single administration, animals should be sacrificed 24 and 48 h later, and only 1000 PCE were scored per animal; additionally, parenteral administration is not physiologically relevant.

Table 1
Genotoxicity profile of perillaldehyde – *in vitro* studies.

Test-system	Test object	Concentration	Results	Reference
Reverse mutation	*S. typhimurium* TA97, TA102	1, 5, 10, 50, and 100 μg/plate	Negative[a,b]	Fujita et al. (1994)
Reverse mutation	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98	Up to 1000 μg/plate	Negative[a,b]	Ishidate et al. (1984)
Mutagenicity	Chinese hamster ovary cells	10, 12.5, 25, 50, and 100 μg/ml	Negative[c,g]	Sasaki et al. (1990)
Mutagenicity	Human fetus cells (Rsa)	0.010 μg/ml	Negative	Suzuki et al. (1990)
	Human fetus cells (Rsa)	0.015 and 0.020 μg/ml	Positive[h]	
Mutagenicity	Human fetus cells (Rsa)	2–200 ng/ml	Positive[i]	Suzuki and Suzuki (1994)
Mutagenicity	*E. coli* WP2	50–400 μg/plate	Negative	Yoo (1986)
Reverse Mutation[a,b]	*S. typhimurium* TA100	1.6, 8, 40, 200, 1000 and 5000 μg/plate	Negative	Bowen (2011)
	S. typhimurium TA102, TA1535, TA1537	0.32, 1.6, 8, 40, 200, 1000 and 5000 μg/plate	Negative	
	S. typhimurium TA98	0.32, 1.6, 8, 40, 200, 1000 and 5000 μg/plate	Positive[c] Negative[e]	
	S. typhimurium TA102, TA1535, TA1537	8.92, 20.48, 51.2, 128, 320, 800, 2000, and 5000 μg/plate	Negative	
	S. typhimurium TA98	8.92, 20.48, 51.2, 128, 320, 800, 2000, and 5000 μg/plate	Positive[c] Negative[e]	
	S. typhimurium TA98[l]	0.32, 1.6, 8, 40, 200, 1000 and 5000 μg/plate	Positive[c]	
hprt assay	mouse lymphoma L5178Y cells	20, 40, 60, 70, 80, 90, and 100 μg/ml[o] 40, 60, 80, 100, 120, 140, 160, and 180 μg/ml[k,p] 20, 40, 50, 60, 70, 80, 90, 100 and 120 μg/ml[o] 25, 50, 75, 100, 120, 140, 160, 170, and 180 μg/ml[p] 4, 8, 12, 15, 18, and 21 μg/mL[q]	Negative[b]	Lloyd (2012)
DNA repair (SOS Chromotest)	*Escherichia coli* PQ37	Not reported	Negative	Eder et al. (1993)
DNA damage (*rec* assay)	*Bacillus subtilis* M45 and H17	0.16, 0.32, and 0.63 μl	Negative	Kuroda et al. (1984)
	Bacillus subtilis M45 and H17	1.25 and 2.5 μl	Positive[n]	
DNA damage (*rec* assay)	*Bacillus subtilis* M45 and H17	2.5 μl/disc	Weak positive	Yoo (1986)
Sister chromatid exchange	Chinese hamster ovary cells	50, 100, 150, and 200 μg/ml	Positive[c,d]	Tayama et al. (1990)
	Chinese hamster ovary cells	50, 100, 150, 200, and 300 μg/ml	Positive[d,e]	
Chromosomal aberration	Chinese hamster fibroblasts	40 and 50 μg/ml	Positive[c,m]	Ishidate et al. (1984)
Chromosomal aberration	Chinese hamster ovary cells	50, 100, 150, 200, and 300 μg/ml	Negative[d,e,f]	Tayama et al. (1990)
	Chinese hamster ovary cells	50, 100, 150, and 200 μg/ml	Negative[c,d,f]	
Micronucleus Induction	Primary human lymphocytes	80, 110, and 120 μg/ml [j,o] 100, 120, and 140 μg/ml[p] 20, 25, and 35 μg/ml[q]	Negative[b]	Lloyd (2009)

[b] With and without metabolic activation.
[a] Pre-incubation with exogenous metabolic system from rat liver.
[c] Without metabolic activation.
[g] Cytotoxic at 12 μg/ml.
[h] Cytotoxic at 0.025 μg/ml.
[i] Cytotoxic at > 20 ng/ml.
[d] Cytotoxic at 150 μg/ml.
[e] With metabolic activation.
[f] Positive only at cytotoxic concentrations.
[j] Cytotoxic ≥ 160 μg/ml.
[k] Cytotoxic ≥ 180 μg/ml (3 h treatment in the presence of S9); cytotoxic ≥ 100 μg/ml (3 h treatment in the absence of S9); cytotoxic ≥ 21 μg/ml (24 h treatment in the absence of S9).
[l] A different batch of test article was used and positive results in TA98 were confirmed.
[m] At 50 μg/ml: 24h and 48h; and at 40 μg/ml: 24h, including polyploidy.
[n] Cytotoxicity at 2.5 ul.
[o] 3 h in the absence of S-9.
[p] 3 h in the presence of S-9.
[q] 24 h in the absence of S-9.

2.2. Genotoxicity testing (since 2010)

Because of the numerous limitations in the earlier genotoxicity studies, new OECD-compliant genotoxicity studies were conducted to establish the mutagenic and genotoxic potential of *p*-mentha-1,8-dien-7-al, including updated mutagenicity studies in bacteria (Ames assays) and in mammalian cells (an *hprt* assay), and a micronucleus assay in human blood lymphocytes. These studies are described in detail in a recent publication (Hobbs et al., 2016), but the data are summarized here to provide a comprehensive overview of the available genotoxicity data for perillaldehyde.

2.2.1. In vitro

An Ames assay was conducted in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102, both in the absence and in the presence of metabolic activation S-9 (from Aroclor 1254-induced rat livers) in three separate experiments (Bowen, 2011; Hobbs et al., 2016). *p*-Mentha-1,8-dien-7-al cytotoxicity was determined in the TA100 strain using the plate incorporation method at concentrations of 1.6, 8, 40, 200, 1000 and 5000 μg/plate, in the presence and absence of S-9. Toxicity was observed at 5000 μg/plate in the absence and presence of S-9 and adequate data were obtained for mutation analysis in strain TA100. *p*-Mentha-1,8-dien-7-al was tested in the other four tester strains at concentrations of 0.32, 1.6, 8, 40, 200, 1000 and 5000 μg/plate, with toxicity observed at 1000 and 5000 μg/plate in all strains with and without S-9, and in a second experiment, at 8.92, 20.48, 51.2, 128, 320, 800, 2000, and 5000 μg/plate, including a 60 min pre-incubation step in the presence of S-9, with toxicity observed in all strains at either 320, 800, or 2000 μg/plate and above in the absence and presence of S-9. Increased revertant frequency was observed in strain TA98 in the absence of S-9 activation in both experiments (at 200 and 1000 μg/plate in the first experiment and at 320 and 800 μg/plate in the second). Other strains did not demonstrate any increases in revertant frequencies. As the result in TA98 was both unexpected and inconsistent with previous Ames assays, the test was repeated in strain TA98 with a different batch of test article but the same treatment conditions. Toxicity was observed at 5000 μg/plate in the absence of S-9 and at all concentrations in the presence of S-9. Unlike the previous two experiments where precipitation was observed on all test plates at 5000 μg/plate, no precipitation was seen at this concentration in the third experiment. However, as in the previous two experiments, a statistically significant and dose-related increase in revertant numbers was observed for strain TA98 at 8 μg/plate and above in the absence but not in the presence of S-9 (Bowen, 2011) (see Table 1).

The mutagenicity of *p*-mentha-1,8-dien-7-al was also tested with the *hprt* assay in mouse lymphoma L5178Y cells to clarify the results of bacterial assays and to assess their relevance for mammalian organisms (Lloyd, 2012; Hobbs et al., 2016). In this assay, the induction of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus was measured in cells treated with *p*-mentha-1,8-dien-7-al at concentrations of 20, 40, 60, 70, 80, 90, and 100 μg/ml for 3 h in the absence of S-9, and 40, 60, 80, 100, 120, 140, 160, and 180 μg/ml in the presence of S-9. No significant increases in mutation frequency or statistically significant linear trends were observed up to toxic concentrations, as relative survival (%RS) dropped to 13% at 100 μg/mL in the absence of S-9 and to 16% at 180 μg/mL in the presence of S-9. In repeat assays, cultures were treated with *p*-mentha-1,8-dien-7-al for 3 h at 20, 40, 50, 60, 70, 80, 90, 100 and 120 μg/ml in the absence of S-9, and at 25, 50, 75, 100, 120, 140, 160, 170 and 180 μg/ml in the presence of S-9, and for 24 h at 4, 8, 12, 15, 18 and 21 μg/mL in the absence of S-9. Relative survival (%RS) decreased to 7% at 120 μg/mL in the absence and to 10% at 180 μg/mL in the presence of S-9 in the 3 h treatments and to 9% in the 24-h treatment. There was no statistically significant increase in mutation frequency under any conditions tested, although a statistically significant linear trend was seen in the absence of S-9 (3- and 24-h treatments) in the repeat assay. However, as this observation was not consistent between the two independent assays and in the absence of any increases in mutant frequencies at any concentration or condition analyzed, the linear trend alone is not considered biologically relevant. Therefore, *p*-mentha-1,8-dien-7-al did not induce mutations in this test system when tested up to toxic concentrations (Lloyd, 2012; Hobbs et al., 2016).

p-Mentha-1,8-dien-7-al was also tested in an *in vitro* micronucleus assay for the induction of chromosome damage in cultured human peripheral blood lymphocytes (whole blood cultures pooled from 2 healthy male volunteers), in the absence and presence of rat liver metabolizing system (S-9), according to OECD Guidelines (Lloyd, 2009; Hobbs et al., 2016). *p*-Mentha-1,8-dien-7-al was added 48 h following culture stimulation by phytohaemagglutinin for either 3 h at concentrations of 80, 110, and 120 μg/ml without S-9, and 100, 120, and 140 μg/ml with S-9, or for 24 h at concentrations of 20, 25, and 35 μg/ml in the absence of S-9. Cytochalasin B (6 μg/ml), to block cytokinesis, was added either at the start of treatment (24-h treatments) or at the start of recovery. Cytotoxicity at the top concentration in each treatment reached 58, 45% and 58%, respectively, i.e., within the target range of 50–60%. There were no statistically significant differences between the frequencies of micronucleated binuclear (MNBN) cells in treated cultures compared to controls; all frequencies were similar to those of concurrent vehicle controls and fell within (or slightly below) historical control ranges. Therefore, *p*-mentha-1,8-dien-7-al was clearly negative for genotoxicity in this assay (Lloyd, 2009). The different outcome between this assay and the earlier positive CA tests may be explained by the fact that human cells but not CHO/CHL are p53-competent, or that different measures of cytotoxicity were used and the extent of cytotoxicity was different. Any concerns would normally be resolved by robust *in vivo* testing for clastogenicity.

2.3. Conclusions from in vitro and older in vivo genotoxicity testing for p-mentha-1,8-dien-7-al

Taken together, inconsistent results were reported in earlier assays, including the sister chromatid exchange assay (SCE), the chromosomal aberration test (CA), rec-assay, and mutagenicity tests. In more recent studies, *p*-mentha-1,8-dien-7-al was

Table 2
Genotoxicity profile of perillaldehyde – *in vivo* studies.

Test system	Test object	Route	Dose mg/kg bw	Result	Reference
Micronucleus assay	Mouse bone marrow cells	i.p.	75, 150, 300, and 600	Negative	Hayashi et al. (1988)
Micronucleus assay	Rat bone marrow cells	oral	175, 350, and 700	Negative	Bowen (2011)
Comet assay	Duodenum	oral	175, 350, and 700	Negative	
	Liver	oral	175, 350, and 700	Negative	

mutagenic in strain TA98 in the absence of S-9 (but negative in the presence of S-9), and negative in the other *Salmonella* strains under all conditions. It was not mutagenic in the *hprt* assay using the mouse lymphoma cell line L5178Y up to toxic concentrations in the presence and absence of S-9. Therefore, the mutagenic effect of *p*-mentha-1,8-dien-7-al seen in TA98 without metabolic activation does not appear to be biologically relevant for mammalian cells. In addition, *p*-mentha-1,8-dien-7-al was negative for chromosomal damage in an earlier *in vivo* MN assay using a single intraperitoneal injection, and in a recent, OECD Guideline-compliant *in vitro* MN assay in human blood lymphocytes. The fact that it is not clastogenic in robust *in vitro* MN assays raises doubts about the relevance of the previously collected *in vitro* CA assays that gave positive results.

In mammals (rabbits and rats), *p*-mentha-1,8-dien-7-al follows metabolic pathways common to other monocyclic (e.g., 2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde) aldehydes that contain alkyl ring substituents, primarily involving oxidation to the corresponding carboxylic acid (e.g. perillic acid), conjugation with glucuronic acid, and excretion in the urine (Ishida et al., 1989; Boon et al., 2000). In a minor pathway, the aldehyde may be reduced to the alcohol which is excreted as the glucuronide conjugate (Ishida et al., 1989; Haag and Gould, 1994). Alternate pathways include reduction of the endocyclic double bond by gut microflora or aromatization of the acid metabolite to yield a benzoic acid derivative (*p*-isopropylbenzoic acid), which may be excreted as the glycine conjugate (Fig. 1) (Ishida et al., 1989). Taking into account the above genotoxicity results and considering the fact that *p*-mentha-1,8-dien-7-al and related substances are rapidly metabolised *in vivo* to compounds of lower toxicological potential, one could reasonably conclude that based on the previously available data and that collected since 2010, *p*-mentha-1,8-dien-7-al is of no concern with respect to genotoxicity.

All the evidence described above was first reviewed by EFSA in 2013 and published in Flavoring Group Evaluation 208 (FGE.208) as part of the re-evaluation of flavoring substances belonging to the chemical group of alicyclic aldehydes with the α,β-unsaturation in ring or side-chain (EFSA, 2013). The EFSA Panel concluded that the overall evidence was insufficient to exclude genotoxicity concerns for *p*-mentha-1,8-dien-7-al and related substances. Remaining doubt and uncertainty, likely driven by the evidence of mutagenicity obtained in the recently conducted Ames assay, led EFSA to request a more definitive study, specifically an *in vivo* comet assay conducted according to OECD Guidelines and with analysis of comet effects in the liver and duodenum.

2.4. Combined in vivo liver and duodenum comet assay and bone marrow micronucleus study

An *in vivo* comet assay was conducted in rats with analysis in the liver as well as in the duodenum (the latter as a first site of contact, and also to address the fact that the results considered positive in the Ames assay were collected in the absence of metabolic activation), in combination with a micronucleus assay with analysis of micronucleus induction in polychromatic erythrocytes (PCE) of the bone marrow (Beevers, 2014; Hobbs et al., 2016). This combination assay was designed to address any remaining concerns for the DNA damaging potential of *p*-mentha-1,8-dien-7-al while also addressing the discrepancy between the results of older assays and the more recent *in vitro* micronucleus assay that was negative (Lloyd, 2009). Young male, out-bred Han Wistar (Crl:WI(Han)) rats (6/dose) were administered *p*-mentha-1,8-dien-7-al by oral gavage at dose levels of 175, 350 and 700 mg/kg bw/day (up to the maximum tolerated dose in males; MTD) on three consecutive days, along with vehicle control and positive (ethyl methanesulfonate; EMS; 150 mg/kg bw) control groups. The top dose induced severe clinical signs of toxicity (decreased activity, ataxia, piloerection and prone posture) in female animals in a range-finding experiment (MTD for females was 500 mg/kg bw/day), but since the difference in toxicity response between males and females was less than 2-fold, the study was conducted in male rats only (as recommended in the OECD Guideline). Signs of toxicity in the main study, indicated by decreased activity in 5/6 animals and ataxia and piloerection in single animals, were noted only at the top dose of 700 mg/kg bw/day. Animals were sacrificed 3h after the last dose administration and were subject to full necropsy. In addition, the study included body weight and clinical chemistry parameters measurement and liver histopathology (see Table 2).

Slightly increased enzyme activities of aspartate aminotransferase and alanine aminotransferase at the highest dose level, indicative of liver injury and consistent with hepatocyte vacuolation noted in histopathological examination confirmed that *p*-mentha-1,8-dien-7-al was tested up to toxic levels. Overall, the study complied with all Guideline validity criteria.

In the micronucleus assay, there was no evidence of bone marrow toxicity, based on the numbers of polychromatic erythrocytes. In addition, no evidence of genotoxicity was found, as statistically significant increases in mean MN PCE frequencies were lacking at any dose level compared to concurrent vehicle controls and all values were within the historical control range. Therefore *p*-mentha-1,8-dien-7-al was clearly negative for induction of micronuclei in the bone marrow of treated rats.

In the comet assay, there was no excessive liver damage (mechanical or necrotic) as indicated by %clouds and %cells with halos, and therefore, the liver toxicity noted above at the top dose did not interfere with the validity of the assay. Group mean tail intensity or tail moment in cells of rats treated with 175 and 350 mg/kg bw/day were comparable to those of the control (150 cells per animal) and all values fell within the historical control range in all treated and control animals. A small but statistically significant increase in the group mean tail intensity was observed at the highest dose level compared to the concurrent control group, although all values remained within the historical control range. This small size of increase and the absence of an effect at the next lower dose suggest an indirect mode of action secondary to tissue toxicity, rather than the result of direct genotoxicity, and therefore subject to a dose threshold for liver toxicity. In the duodenum, there were no

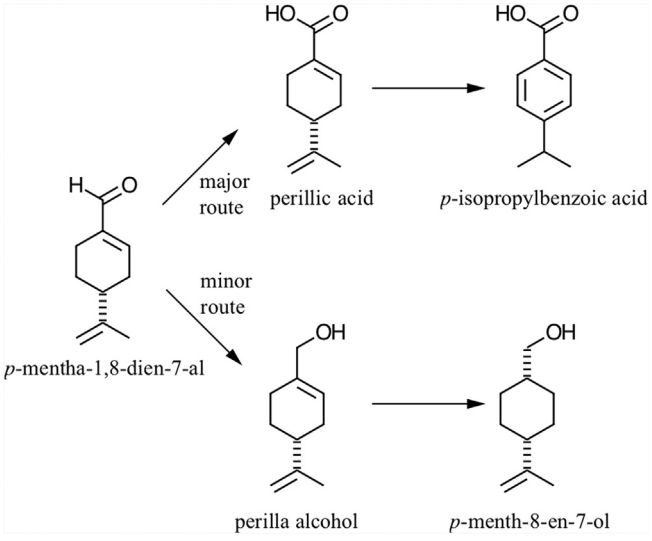

Fig. 1. Recognized metabolism of *p*-mentha-1,8-dien-7-al in mammals.

statistically significant increases in the mean tail intensities and all values were within the historical control range. This result directly addresses uncertainties about small increases in genotoxicity assays in the absence of metabolic activation, since it reflects the direct action of *p*-mentha-1,8-dien-7-al at the site of contact.

3. Criteria of assessment for the *in vivo* comet assay

According to OECD TG 489 guidelines, paragraph 59 (clearly positive results), a positive result in the comet assay requires all acceptability criteria to be met and when not, paragraph 62 (neither clearly negative nor positive) offers some guidance (OECD TG 489):

> *"Evaluation and Interpretation of Results.*
>
> *(Paragraph 59) Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if:*
>
> *a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control,*
>
> *b) the increase is dose-related when evaluated with an appropriate trend test,*
>
> *c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations.*
>
> *When all of these criteria are met, the test chemical is then considered able to induce DNA strand breakage in the tissues studied in this test system. If only one or two of these criteria are satisfied, see paragraph 62.*
>
> *(Paragraph 62) In case the response is neither clearly negative nor clearly positive (i.e. not all the criteria listed in paragraphs 59 or 60[2] are met) and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations conducted, if scientifically justified. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using optimised experimental conditions (e.g. dose spacing, other routes of administration, other sampling times or other tissues) could be useful."*

Further to the above criteria, the biological relevance of results is especially critical to assess in light of positive or equivocal results, as stated in the Guideline:

> *"To assess the biological relevance of a positive or equivocal result, information on cytotoxicity at the target tissue is required (see paragraphs 54–55). Where positive or equivocal findings are observed solely in the presence of clear evidence of cytotoxicity, the study would be concluded as equivocal for genotoxicity unless there is enough information that is supportive of a definitive conclusion."*

Indeed, the EFSA Scientific Committee (EFSA, 2011) and others (ICH, 2011) recommend a "weight-of-evidence" approach to reach a complete and accurate evaluation and interpretation of genotoxicity data. The weight-of-evidence approach considers not only the quality and reliability of the genotoxicity assay data, but also any relevant data, including physicochemical properties, structure-activity relationships and 'read-across' from structurally related substances, bioavailability, toxicokinetics and metabolism, and evidence from chronic repeated-dose toxicity and carcinogenicity studies. In this context, the FEMA Expert Panel suggests that three critical questions must be considered for the correct interpretation of test results within the overall body of evidence and adherence to Guideline criteria:

- Is there evidence for a consistent biological mechanism of genotoxicity among assays?
- Are the effects reproducible?
- Is the evidence obtained from genotoxicity assays biologically relevant within the broader body of evidence (e.g. outcomes of carcinogenicity studies)?

These questions are similar to the criteria in the ICH Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2011), and have been considered relevant to serve as a guide in the discussion that follows regarding the different interpretation of the results of the most recent *in vivo* genotoxicity study (Beevers, 2014) within the overall weight-of-evidence of genotoxicity for *p*-mentha-1,8-dien-7-al.

4. Recently published EFSA opinion on the genotoxicity of *p*-mentha-1,8-dien-7-al

The comet/micronucleus combination assay for *p*-mentha-1,8-dien-7-al was recently reviewed by the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), and the result of the evaluation was published in a recent Scientific Opinion (EFSA, 2015). The Panel concluded that there is a safety concern for its use as flavoring substance based on the positive finding in the liver comet assay. In addition, since *p*-mentha-1,8-dien-7-al was considered as a 'representative' substance for 10 other substances, it was also concluded that there is a potential safety concern for the other substances as well.

EFSA issued a press release (23/07/2015) entitled "Flavoring substance considered a safety concern," stating:

> "*The flavouring substance p-mentha-1,8-dien-7-al (also called "Perilla aldehyde") has been shown to be genotoxic (damaging to DNA) in a new study in animals, evaluated by EFSA's experts. Under EFSA's process for the safety assessment of food flavourings, once a substance is characterised as a genotoxin, EFSA concludes on this aspect only without taking into account consumer exposure*."

This conclusion reached by EFSA appears to be inconsistent with the OECD Guideline criteria for interpretation of results of a comet assay as described earlier. The Panel correctly concluded that the other genotoxicity assays did not provide any evidence of genotoxicity for *p*-mentha-1,8-dien-7-al. The final determination by EFSA that *p*-mentha-1,8-dien-7-al is genotoxic was reached on the basis of two of the three criteria for a positive test, namely the statistically significant difference between one treatment group from its concurrent control group, and statistical evidence of dose-response. A thorough and detailed examination of the scientific data presented to the EFSA Panel leads one to a different conclusion if the interpretation criteria were to be applied as intended.

More specifically, a key factor in the interpretation of the results of the comet assay in the liver is the evidence of general toxicity and hepatotoxicity at the highest dose tested. The lack of dose-related increases in % clouds or % cells with halos in liver cells indicates that treatment did not cause *excessive* DNA damage that could have created artefacts and interfered with comet analysis. However, other endpoints reveal evidence of dose-dependent general liver toxicity in the test substance-exposed animals, and this effect was particularly pronounced at the highest dose employed (700 mg/kg bw/d), including a loss of body weight in the high dose group

[2] Note: paragraph 60 lists criteria that must be met for a clearly negative result.

over the period of exposure to the test substance in 5 of the 6 rats in the group, elevated aspartate aminotransferase and alanine aminotransferase and altered clinical biochemistry parameters (cholesterol, potassium, chloride, urea and glucose); three animals (numbered 27, 23 and 22) in the high dose group were particularly affected. Histopathological examination (evaluated with identity of group blinded) corroborated the clinical pathology findings with observations of hepatocyte vacuolation in all 6 animals in the high dose group. Additionally, 5 of the 6 animals in the high dose group showed overt signs of toxicity reflected in their behavior (reduced activity), particularly animals 27 and 23. The observed toxicity *in vivo* is consistent with dose-dependent cytotoxicity of *p*-mentha-1,8-dien-7-al seen in previously reported *in vitro* genotoxicity tests, in the presence and absence of a metabolic activation system. The dose-dependent cytotoxicity of *p*-mentha-1,8-dien-7-al was a recurring characteristic of the material and was a limiting factor in a number of these assays.

With regards to the parameters of the comet assay, interpretation of the results is guided by the three criteria set forth in the OECD Guideline:

> *a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control*" (OECD, 2014)

The EFSA Panel concluded that

> "*at the highest dose a 3.4-fold and statistically significant increase in tail intensity was observed. Five out of the six animals treated with the highest dose had tail intensities that exceeded the values observed in the concurrent vehicle control animals*" (EFSA, 2015)

While this statement is correct, it should be pointed out that only 2 of the 6 animals in this dose group, are driving the statistically significant increase in group mean tail intensity, compared to the concurrent vehicle control animals. More importantly, these two animals were also among the three most affected by liver toxicity (animals 27 and 23), indicating a direct association between liver toxicity and increased DNA tail intensities.

> *b) the increase is dose-related when evaluated with an appropriate trend test*,

The EFSA Panel stated that

> "*A statistically significant linear trend was also apparent.*" (EFSA, 2015)

While this statement is also accurate, this is also consistent with the reported dose-dependent toxicity.

> *c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations*. (OECD, 2014)

The EFSA Panel stated that

> *"… however, the tail intensity values for all animals fell within the laboratory's historical control values."*

The observation that tail intensity values for all animals fell within the laboratory's historical control values is pertinent and under OECD TG 489 guidelines cannot be dismissed when considering the outcome of a comet assay.

Additional elements of the study bear weight on the interpretation of the results. The EFSA Panel noted that

> *"the range for both the negative and positive historical control values were extremely wide for this test laboratory. In addition, there was an overlap of the negative (95% range: 0.02–11.39) and positive (95% range: 7.15–65.07) control values."* (EFSA, 2015)

This is indeed the case, but it is important to consider that the tail intensity for the negative and positive controls of the specific assay fell comfortably within the range of the historical control values and near the means of the respective ranges: the positive control tail intensity is close to the historical positive control mean (35.55 ± 14.86). More importantly, the mean tail intensity of the high dose group (700 mg/kg bw/d) of 2.20 ± 0.60 is comparable to the historical negative control mean (2.22 ± 2.58), *despite* the skew effected by animals 27 and 23.

5. Conclusions

Overall, the pattern of DNA damage reported is described as a statistically significant increase in mean tail intensity only at the high dose (compared to concurrent control) and a statistically significant linear trend, but also as a small scale of mean tail intensity increase at the highest dose relative to the concurrent control, a mean tail intensity in the low and middle dose groups similar to the average of the historical control range, a mean tail intensity driven by two animals in the group, and direct correlation with clear biochemical and histopathological evidence of liver toxicity. This pattern is consistent with a mode of action of DNA damage secondary to cytotoxicity. Therefore, the final conclusion the EFSA Panel reached that "*The comet arm of this study indicates that p-mentha-1,8-dien-7-al induces DNA damage in liver*" does not follow the criteria as set in the OECD Guideline. First, it violates the criteria for a positive test according to the Guideline, since the third criterion for a positive comet assay has not been met and therefore expert judgement is paramount. Second, where expert judgement was called for, this conclusion dismissed the impact of toxicity on the comet assay finding and lost sight of the absence of biological relevance. It is clear that the positive result in the comet assay is confounded by toxicity associated with treatment, particularly evident at the high dose (700 mg/kg bw/d). According to OECD TG 489 guidelines, *"it is not possible to distinguish DNA migration induced by genotoxicity versus that induced by cytotoxicity in the comet assay alone*" and "*Increases in DNA migration in the presence of clear evidence of cytotoxicity should be interpreted with caution*" (paragraph 54, OECD TG 489). In cases of confounded results the Guideline proposes that "*Changes in clinical chemistry measures (e.g. AST, ALT), can also provide useful information on tissue damage and additional indicators such as caspase activation, TUNEL stain, Annexin V stain, etc. may also be considered*" (paragraph 55, OECD TG 489). In fact, the two animals (numbered 27 and 23) that drove the significant increase in tail intensity are also those with the most pronounced evidence of hepatotoxicity.

In the view of the FEMA Expert Panel, any disregard for consideration of the laboratory historical controls for this assay, and for interpreting the data outside of the OECD guidelines is not appropriate nor justified. For the reasons described above, we concur with the conclusion of the authors of the comet assay report (reproduced below for clarity) and agree that it is consistent with the data and consonant with the OECD TG 489 Guideline:

> "*It is concluded that under the conditions of this study, p-Mentha-1,8-dien-7-al did not induce an increase in micronucleated polychromatic erythrocytes of the bone marrow of male rats following*

oral gavage administration of doses up to 700 mg/kg/day (an estimate of the maximum tolerated dose for this study). A small but statistically significant increase in DNA damage in the liver was detected in the comet assay following treatment with p-mentha-1,8-dien-7-al at 700 mg/kg/day. However, as this increase was concomitant with changes in liver enzymes and evidence of perturbation of hepatocyte function, the DNA damage may be due to a mechanism other than genotoxicity. No DNA migration was observed [at] 175 or 350 mg/kg/day, where there was also no evidence of liver toxicity."

This assessment by the study authors is consistent with the interpretation of the results by the FEMA Panel and not aligned with that of EFSA, suggesting a possible need for re-evaluation of the study to ensure that the OECD TG 489 Guideline is taken into full account when the study is interpreted.

Author contributions

FEMA Expert Panel Members Cohen, Fukushima, Gooderham, Guengerich, Hecht, Rietjens, and Smith interpreted the results and drafted the manuscript. Ms. Harman, Drs. Bastaki, McGowen, and Taylor researched and compiled available study data and assisted in the drafting of the manuscript.

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Transparency document

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