Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol

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Eugenol (1-allyl-3-methoxy-4-hydroxybenzene; CAS No. 97-53-0), a compound extracted from clove oil and marjoram, is widely used as a food flavouring substance and is present in spices such as basil, cinnamon and nutmeg. It is also used in dentistry as an antiseptic and analgesic. Structural similarities with the class IIB IARC carcinogen safrole raises questions on its putative carcinogenicity. We evaluated the genotoxicity of eugenol in V79 cells using chromosomal aberrations (CAs), with and without rat liver biotransformation (S9). Eugenol induced CAs, with significant increases (3.5% aberrant cells) at 2500 M, demonstrating cytotoxicity at higher doses. S9 increased the induction of CAs in a dose-dependent manner to 15% at 2500 M, with a high frequency of chromatid exchanges. In particular, an increase of endoreduplicated cells was observed, from 0% at control levels to 2.3 and 5% at 2000 M, without and with S9, respectively. Since endoreduplication has been linked to inhibition of topoisomerase II, the topoisomerase II inhibitor ICRF-193 was used as a control inducer of endoreduplication (0.1–0.5 M), increasing the number of endoreduplicated cells from 0% (control) to 3.5% (0.5 M). S9 did not influence endoreduplication by ICRF-193. Both eugenol and ICRF-193 were also assayed for inhibition of topoisomerase II, and both showed a dose-dependent inhibitory effect, with ICRF-193 being a more potent inhibitor. Our results confirm that eugenol is genotoxic and raises the possibility of it having topoisomerase II inhibiting activity.

Introduction

The large-scale use of certain food flavourings requires accumulation of toxicological data on these substances, particularly in cases where structural similarities with other known substances showing genotoxic or carcinogenic properties indicate that restrictions on human consumption or exposure should be implemented (1–3). This is the case of the flavouring substance eugenol, structurally similar to safrole, a class 2B IARC carcinogen. In all these cases human exposure is widespread through consumption of food and beverages, raising the possibility of adverse effects in human populations (4). Eugenol is present in a variety of essential oils, such as clove oil (85–95% eugenol), marjoram essential oil (10%), ground cinnamon (0.02%), ground cloves (1%), clove oleoresin (60–90%) and in cinnamon leaf oil (70–90%) (5). Eugenol is also used widely as an analgesic and antiseptic in clinical dentistry. The yearly worldwide production of eugenol is estimated at ~22 000 kg (6), and it is used mostly by the food, perfume and pesticide industry (7).

The average maximum used levels in food products, such as beverages, ice cream, baked goods, gelatin and puddings, and chewing gums, range from 1.4 to 500 p.p.m. (5). Although the acceptable daily intake (ADI) was revised by the International Programme on Chemical Safety in 1982 to 0–2.5 mg/kg bw (8), estimates of average human consumption of eugenol vary from 7 to 76 μg/day (6). No further evaluation has been performed by major health risk organizations. Eugenol is a flavouring substance authorized by the European Commission for use in foodstuffs, although no ADI was established by the Commission. This is in contrast to other flavourings for which an ADI has been calculated or no safe exposure limit could be established.

Although eugenol’s toxicity has been studied in laboratory animals (5), little or no information is available on human exposure and subsequent possible adverse health outcomes. Both toxicological and human exposure data are needed to make accurate risk evaluations. From a chemical point of view, eugenol is a alkenylbenzene derivative, similar to estragole, methyleugenol and safrole. Owing to its structural similarity to the carcinogen safrole, there is concern about its carcinogenic and mutagenic properties (9). The carcinogenicity of eugenol was evaluated by the US National Toxicology Program (NTP) with negative outcomes for F344 rats of either sex, whereas results for male and female B6C3F1 mice were considered equivocal (5). As for its genotoxicity, results were negative in various Salmonella typhimurium strains, either with or without metabolic activation. Results in other test systems showed genotoxic activity in Chinese hamster ovary cells, using chromosomal aberrations (CAs) as endpoints (10). Recently Hikiba et al. (11) evaluated the genotoxicity of eugenol together with other chemicals used in dental practice and observed an induction of CAs in Syrian hamster embryo cells. The presence of exogenous metabolic activation enhanced the genotoxicity of eugenol. The Drosophila wing somatic mutation and recombination test (SMART) was used to evaluate the recombinogenic activity of eugenol, with positive results in a variant having increased cytochrome P450 activity (12), once again emphasizing the need for metabolic activation for increased genotoxicity.

Accumulated evidence suggests that the biotransformation of eugenol should be similar to the biotransformation of other alkenylbenzene derivatives such as estragole and methyleugenol (9,13–18). Thus, eugenol would undergo three metabolic reactions, hydroxylation, epoxidation and O-demethylation. The reactive metabolite, 2,3-epoxyeugenol, after originating the quinone methide, can
react with DNA, forming adducts (13,16,18–20) that can contribute to the genotoxic activity of eugenol.

Following our interest on the mechanisms of genotoxicity of food compounds (3,21–23), we report here results on the genotoxicity of eugenol in V79 Chinese hamster cells, with and without exogenous biotransformation, using CAs as an endpoint. The observation that eugenol induced endoreduplication (ER) in these cells led us to evaluate this endpoint, both with and without exogenous biotransformation. Since ER has been linked to topoisomerase II (topo II) inhibition (24), the bis-2,6-dioxopiperazine derivative ICRF-193 was used both as a reference inhibitor of topo II and an inducer of ER (25).

Material and methods

Chemicals and culture media

Eugenol (99%), Mitomycin C (MMC), newborn calf serum and Ham’s F-10 medium, cytochalasin-B, penicillin and streptomycin, 5-bromo-2-deoxiuridine, cyclophosphamide (CP) were purchased from Sigma (St Louis, MO). Dimethylsulfoxide (DMSO), methanol, acetic acid, potassium benzimidazol and cyclophosphamide (CP) were purchased from Trinova Biochem GmbH (Giessen, Alemanha). Colchicine was purchased from Fluka (Buchs, Switzerland). Trypsin was obtained from Difco Laboratories (Detroit, Mich). ICRF-193 (meso-2,3-bis(3,5-dioxopiperazine-1-yl)butane) was purchased from Zenyaku Kogyo Co, Japan. Acorol 1354 induced rat liver S9 fractions were purchased from Trinova Biochem GmbH (Giessen, Alemania).

V79 cells CAs assay

V79 Chinese hamster cells were cultured in 5 ml Ham’s F-10 medium supplemented with 10% newborn calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and incubated at 37 °C in an atmosphere containing 5% carbon dioxide.

Twenty-four hour cultures (~5 × 10⁶ cells/flask) growing as monolayers in 25 cm² tissue culture flasks (Greiner; Frickenhausen, Germany) were treated with different doses of eugenol (100–3000 μM) or ICRF-193 (0.1–0.5 μM), both dissolved in DMSO, for 3 h. When testing in the presence of a biotransformation system, 500 μl of 10% S9 Mix, prepared as described previously (26), was added to the flasks. In all the experiments, the cells were then washed and incubated with fresh medium for an additional period of 13 h. Colchicine was then added at a final concentration of 6.5 mM. Cells were fixed for a further 3 h and then harvested by trypsinization.

Table I. Eugenol induced a dose-dependent increase of aberrant cells (Table I). Multi-aberrant cells are defined as cells containing more than 10 CAs, excluding gaps. These cells are included as CAs in an atmosphere containing 5% carbon dioxide.

Topo II activity

Topo II activity was assayed using a TopoGen (Columbus, OH) assay kit based on the decatenation of supercoiled plasmid. The pRYG is a plasmid containing a single, high affinity topo II cleavage and recognition site. The amount of DNA substrate (pRYG) used in each assay was 0.04 pmol (27). Immediately before use, test and control compounds were diluted in 1% (v/v) DMSO to maintain DMSO concentration at or below 1% of the final incubation volume. Eugenol was added to the reaction Mix at concentrations of 5–7500 μM and ICRF-193 was used as positive control at concentrations of 1–10 μM. After 60 min incubation at 37 °C, the samples were loaded onto 1% agarose gel and subjected to electrophoresis for 1.30 h at 100 V/cm. Finally the gel was stained (45 min) with 0.5 μg/ml ethidium bromide, destained in distilled water (10 min) and photographed with FujiFilm S500 digital camera.

Results

Results of CAs induced by eugenol in V79 cells are shown in Table I. Eugenol induced a dose-dependent increase of aberrant cells excluding gaps (ACEG) in V79 cells up to 3000 μM, in the presence of S9 mix, with significant increases from 0.5% (control) to 18% (3000 μM) with particular emphasis on chromatid exchanges. In the absence of S9 mix, however, the genotoxicity of eugenol was not as clear, although there was an increase in aberrant cells from 0% (control) to 3.5% aberrant cells at 2500 μM, and 12% at 3000 μM, demonstrating cytotoxicity at higher doses, as indicated by the mitotic index (Table I). Multi-aberrant cells are defined as cells containing more than 10 CAs, excluding gaps. These cells are included...
The observation that eugenol induced ER led us to evaluate this endpoint, with and without S9 mix. The results obtained for endoreduplicated cells are shown in Tables II and III, and Figure 1. As can be seen, eugenol induced a dose-dependent increase in the percentage of endoreduplicated cells, in the absence of rat liver biotransformation system S9. An increase in endoreduplicated cells was observed, from 0% at control levels to 2.2% at 2000 μM (Table II), which increased in the presence of S9 (5% at 2000 μM) (Table III). At 2500 μM high levels of cytotoxicity were observed. The increase in ER in the presence of S9 mix once again suggests a contribution of biotransformation enzymes to the ER inducing activity of eugenol. As a positive control inducer of ER, the alkenylbenzene derivative ICRF-193 was chosen, which also induced a dose-dependent increase in endoreduplicated cells, as expected (28). These results are presented in Tables II and III and shown in Figure 2. ICRF-193 is a more potent inducer of ER than eugenol. The presence of the rat liver S9 did not alter the endoreduplicating inducing activity of ICRF-193.

Since ER has been linked to inhibition of the enzyme topo II, the activity of this enzyme was assayed in the presence of eugenol. The alkenylbenzene derivative ICRF-193 was used as a positive control. The results show that eugenol inhibits
topo II in a dose-dependent way, albeit at concentrations well above ICRF-193 (Figure 3).

Discussion

Eugenol is a widely used flavouring substance present in various foodstuffs but also finds application in cosmetics and perfumes. Data collected on eugenol has shown that it is genotoxic in various test systems e.g. inducing CAs in eucaryotic cells (5,11) and mutations and recombinations in the Drosophila wing SMART test (12). Concern about the carcinogenicity of eugenol has risen mainly because of structural similarities with the alkenylbenzene safrole, which is classified by IARC as possibly carcinogenic to humans (29), even though carcinogenicity studies by the NTP revealed equivocal results in B6C3F1 male or female mice (5).

Two other food alkenylbenzenes, estragole and methyl-epi-safrole, have been evaluated for their safety by the European Union Scientific Committee on Food (30,31), classifying them as genotoxic, indicating the need for restrictions on consumption. In contrast, the industrial expert panel from the Flavour and Extract Manufacturers Association (FEMA) considered that exposure to methyleugenol and estragole does not pose a significant threat to humans (13). This conclusion is based on mechanistic insight on the biotransformation of these alkenylbenzenes and the mechanisms of genotoxicity. Thus, according to the FEMA expert panel, the profiles of metabolism, bioactivation and covalent binding of these flavours are dose dependent, with a marked decrease at lower exposure doses (1–10 mg/kg) that are 100–1000 times higher than calculated or anticipated human exposure (13).

Owing to structural similarities, the mechanisms of genotoxicity of eugenol may show similarities with those of estragole and methyleugenol. Nevertheless, these mechanisms are not fully known. In these studies we demonstrated that eugenol induces chromosome aberrations, including exchanges, in V79 cells, in particular in the presence of rat liver S9 mix, which suggests biotransformation to reactive metabolites, in agreement with published reports (11,14,19). Eugenol is known to undergo at least two major biotransformation reactions, epoxidation leading to 2,3'-epoxieugenol and hydroxylation with generation of 1'-hydroxyeugenol and subsequently leading to the formation of the reactive metabolite quinone methide. This metabolite is highly reactive, rapidly forming DNA adducts (19), indicated to contribute to the induction of CAs in V79 cells (13,16,18,19). Since V79 cells are known to be devoid of cytochrome P450 activity (32,33), the genotoxicity results in the absence of S9 mix could be due to either the direct acting activity of eugenol or possibly through formation of reactive oxygen species (20,34). The fact that 8-hydroxy-2'-deoxyguanosine can be produced by eugenol lends credence to this latter hypothesis (19).

Regarding CAs, we observed essentially the formation of chromatid exchanges (triradial and quadradiral figures), indicating recombination phenomena that could indicate other biological activities of eugenol or its metabolites. Published data has indicated a potential for eugenol being recombinogenic (12). From our results, an interesting observation was the induction of ERs induced by eugenol, which increased in a dose-dependent manner (Figure 1), which is potentiated in the presence of S9 mix. ER is a rare phenomenon observed in animal cells, frequently induced by chemicals, typically inhibitors of topo II (28,35–39), entailing two successive rounds of DNA replication without segregation of daughter chromatids, giving rise to diplochromosomes. The mechanisms involved in the induction of ER are not known, but probably involve DNA damage, cytoskeleton disturbance or topo II inhibition and certainly include disruption of cellular checkpoint controls (36).

Topo II is an essential cellular enzyme that catalyses changes in DNA topology via its cleavage–religation equilibrium, allowing decatenation of catenated DNA, which is fundamental in various cellular processes, including DNA replication and cell division [see review by Larsen et al. (40)]. Agents that are capable of stabilizing the DNA-topo II complex, also known as the cleavable complex, are called topo II poisons, whereas agents that act on other steps of the enzyme’s catalytic cycle are called catalytic inhibitors (40). Topo II-targeted drugs that are poisons of this enzyme stabilize topo II–DNA covalent complexes, leading to chromosome breaks. The finding that inhibitors of topo II could induce ER (37,39,41) led us to evaluate the inhibitory activity of eugenol.

![Fig. 2. Endoreduplicating inducing activity of ICRF-193 in V79 cells. Values are percentage of endoreduplicated cells scored in 2000 metaphases, presented as averages and standard deviations.](https://academic.oup.com/mutage/article-abstract/21/3/199/1132927)

![Fig. 3. Topo II activity in presence of eugenol and ICRF-193. Lanes 1–15: pRYG (0.04 pmol); lane 1: with Human Topo IIα (1 unit); lane 2: without enzyme; lane 3: with enzyme and DMSO; lanes 4–12: with enzyme and eugenol (5; 10; 50; 100; 500; 1000; 2500; 5000 and 7500 μM, respectively); and lanes 13–15: with enzyme and ICRF-193 (1; 5 and 10 μM, respectively).](https://academic.oup.com/mutage/article-abstract/21/3/199/1132927)
towards topo II. Our results (Figure 3) showed that eugenol does, indeed, inhibit the catalytic activity of topo II. Comparison of the inhibitory activity of eugenol with that of an established topo II inhibitor, ICRF-193, revealed that the latter is more potent than eugenol. ICRF-193 was chosen as a reference inhibitor since it is a known topo II inhibitor and also induced ER (28). Our results confirm the endoreduplicating activity of ICRF-193, in V79 cells. Interestingly, the relative concentrations of eugenol and ICRF-193 required to induce ER seems to be maintained when evaluating topo II inhibition (approximately 5000-fold difference). Lynch et al. (42) have observed that the topo II inhibitors etoposide, doxorubicin, ciprofloxacin and genestein exhibited in vitro thresholds for clastogenicity using L5178Y mouse lymphoma cells. However, these cell lines are transformed and p53 deficient. The authors of the work stated that the relevance of their observations to a genotoxic risk assessment is uncertain, as are the concentrations that define the threshold for the topo II inhibitors. Thus, caution is needed in considering a threshold approach for eugenol and related compounds since they have been shown to be genotoxic in various assays.

Former reports have indicated an increase in ER associated with a deficiency in various DNA repair genes, including the X RCC3 gene, important in recombination processes (43). The Chinese hamster cell line EM9 is a repair deficient mutant that shows elevated indices of ER (28) and a 10-fold higher baseline frequency of SCEs relative to the parental cell line AA8 and also a higher sensitivity to killing by X rays. This defect is corrected by the human XRCC1 gene (44), once again, implicating DNA repair genes in its phenotype. Thus, further studies are required to evaluate the endoreduplicating activity of eugenol in other cell lines such as EM9 and topo II inhibition in vivo. In conclusion, in this report we confirm the genotoxicity of eugenol and the importance of biotransformation in its genotoxicity, in particular the induction of ER, raising the possibility of eugenol being an inhibitor of topo II.

References


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