

Skin sensitization potency of isoeugenol and its dimers evaluated by a non-radioisotopic modification of the local lymph node assay and guinea pig maximization test

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ABSTRACT: Allergic contact dermatitis is the serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with spicy, carnation-like scent, is used in many kinds of cosmetics and is a well-known moderate human sensitizer. It was previously reported that the dimerization of eugenol yielded two types of dimer possessing different sensitization potencies. This study reports the differences in skin sensitization potencies for isoeugenol and two types of dimer, β -O-4-dilignol and dehydrodiisoeugenol (DIEG), as evaluated by the non-radioisotopic local lymph node assay (non-RI LLNA) and guinea pig maximization test. In the guinea pig maximization test, isoeugenol, β -O-4-dilignol and DIEG were classified as extreme, weak and moderate sensitizers, respectively. As for the results of non-RI LLNA, the EC₃ for isoeugenol, β -O-4-dilignol and DIEG were calculated as 12.7%, >30% and 9.4%, respectively. The two types of isoeugenol dimer showed different sensitizing activities similar to the case for eugenol dimers. A reduction of sensitization potency achieved by dimerization may lead to developing safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, the dimerization of isoeugenol may yield a promising candidate as a cosmetic ingredient with low sensitization risk. The data may also provide useful information for the structure-activity relationship (SAR) in skin sensitization. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: isoeugenol; isoeugenol-dimer; local lymph node assay; guinea pig maximization test

Introduction

Allergic contact dermatitis is a serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with a spicy, carnation-like scent, and is used in many kinds of cosmetics (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). However, isoeugenol is a well-known moderate human sensitizer of human potency class II, and its sensitizing activity was confirmed in mice, guinea pigs and humans (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). Eugenol is also known as a human sensitizer and is used in various consumer products similar to isoeugenol (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). There are many publications concerning the sensitization potential of eugenol monomer, however, the sensitization potentials of eugenol dimers which are

formed through its auto-oxidation process were not known. Recently the sensitization potential of eugenol dimers was investigated and it was found that the dimerization of eugenol, a moderate human skin sensitizer, yielded two types of dimer possessing different sensitization potencies, namely 2,2'-dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl as a weak sensitizer and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxy phenyl ether as an extreme sensitizer (Takeyoshi *et al.*, 2004).

Eugenol and isoeugenol are both important cosmetic ingredients with whitening and antibacterial effects (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). A reduction of sensitization potency achieved by dimerization may lead to the development of safer cosmetic ingredients. As in the case of eugenol, dimerization of isoeugenol yields two types of isoeugenol dimer. These isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and anti-bacterial effects similar to the monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for a cosmetic ingredient with low sensitization risk.

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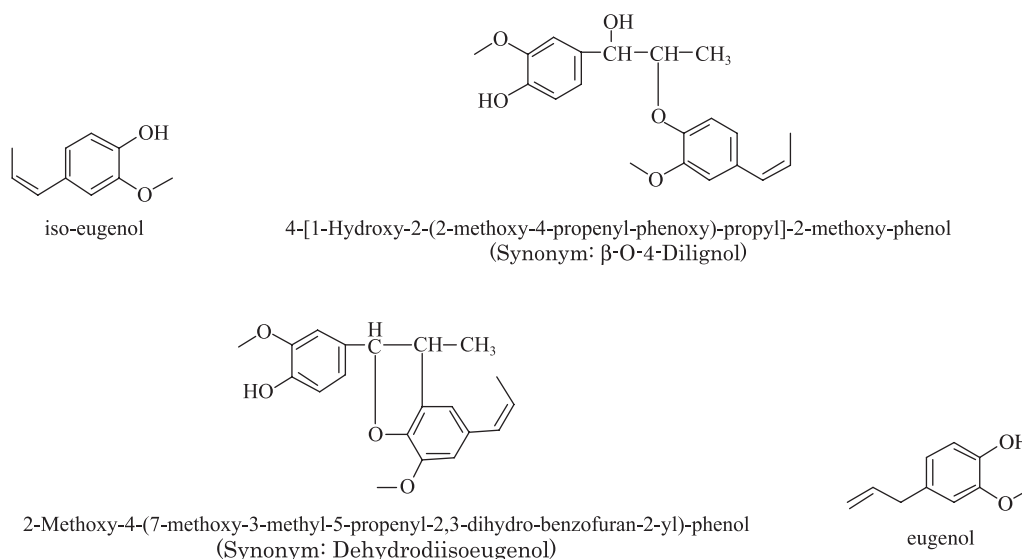


Figure 1. Chemical structures of isoeugenol, isoeugenol dimers and eugenol used in this study

This study employed two types of sensitization assay, i.e. a conventional guinea pig maximization test and non-radioisotopic local lymph node assay (non-RI LLNA) which can provide quantitative outcomes (Takeyoshi *et al.*, 2004) for evaluating the sensitization potency of related compounds. The possibility of a reduction of sensitization potency by dimerization of isoeugenol monomer was investigated.

Materials and Methods

Chemicals and Reagents

Isoeugenol and its dimers, β -O-4-dilignol and dehydrodiisoeugenol, were provided by Kanebo cosmetics, Inc. (Fig. 1, Kanagawa, Japan). Eugenol was obtained from Aldrich Chemicals Inc. All chemicals were confirmed to have >98% purity according to the HPLC analysis and were dissolved in olive oil for the GPMT, or in acetone:olive oil (AOO; 4:1) for the non-RI LLNA. 5-Bromo-2'-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg ml⁻¹.

Animals

Female Hartley guinea pigs and CBA/JN strain mice were obtained from SLC Japan Ltd (Shizuoka, Japan) and Charles River Japan Ltd (Kanagawa, Japan), respectively. The animals were housed in animal rooms maintained at a temperature of 22 $\pm$ 3 $^{\circ}$ C, and at a relative humidity of 55 $\pm$ 15%. The rooms were ventilated at a frequency of 10–15 cycles h⁻¹, and lighted artificially

for 12 h daily. Animals were allowed free access to laboratory diet (RC-4 for guinea pigs and MF for mice, Oriental Yeast Co., Tokyo, Japan) and tap water.

Guinea Pig Maximization Test (GPMT)

Guinea pigs were allocated randomly to three groups (10 animals/group). The test was conducted according to a method described previously (Magnusson and Kligman, 1969). Guinea pigs received a series of intradermal injections of eugenol, or of its dimers, in the shoulder region to induce sensitization. After 6–8 days, sensitization was boosted by a 48 h occluded patch of the same compound placed over the injection sites. Then 14 days later, the animals were challenged on a shaved flank by a 24 h occluded patch containing the same compound. All induction and challenge concentrations were set at 5% (maximum non-irritant concentration) in olive oil for all compounds as a result of preliminary dose finding tests. All compounds elicited apparent irritation at 10% in preliminary tests for intradermal injection and topical application, and then it was decided to use induction and challenge concentrations of 5% for all compounds to compare the sensitization potency of these three compounds. Chemicals were classified by the sensitization rate for each chemical, 0%–8%: weak, 9%–28%: mild, 29%–64%: moderate, 65%–80%: strong, 81%–100%: extreme.

Non-radioisotopic Local Lymph Node Assay (Non-RI LLNA)

The assay was conducted according to a method described previously (Takeyoshi *et al.*, 2001). Mice were

allocated randomly to 12 groups (4 animals/group). A 25 µl volume of test chemicals at concentrations of 3%, 10% or 30% was applied to the dorsum of both ears of the mice, daily for 3 consecutive days. The concentration ranges of each test chemical were decided according to the sensitization potencies classified by the results of GPMT. A single intraperitoneal injection (5 mg per mouse) of BrdU was then given on day 4. On day 5, the draining auricular lymph nodes were removed, weighed and stored at -20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure the BrdU incorporation. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit (Roche Applied Science Corp., Indianapolis, IN, USA; Cat. No. 1647229). The lymph nodes were crushed, passed through a 70 µm nylon mesh, and the lymph node cells were suspended in 15 ml of physiological saline individually. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate in triplicate. After centrifugation (300 g, 10 min), the supernatants were removed. A 200 µl volume of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well, and after rinsing three times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index and the stimulation index (SI) which is the ratio of individual labeling index of each animal against the mean labeling index for the concurrent vehicle control group was calculated. Then the estimated concentration of a chemical required to induce a stimulation index of 3 relative to vehicle-treated controls (EC3 value) was derived by linear interpolation as described previously (Basketter *et al.*, 2000). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI

value of 3 have the coordinates (*a*, *b*) and (*c*, *d*) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

Statistical Analysis

The mean labeling index was calculated for vehicle control group. The stimulation index (SI) defined as a relative labeling index of the test group to the vehicle control value was then calculated. Data were analysed using a one-way analysis of variance (one-way ANOVA). If the one-way ANOVA produced a significant difference, the differences between the concurrent vehicle control group and each of the experimental groups were analysed using the Dunnett's multiple comparison tests (Bruning and Kintz, 1997).

Results

Guinea Pig Maximization Test (GPMT)

In the guinea pig maximization test for isoeugenol and its dimers, the sensitization response rates were as follows: isoeugenol 100%, β-O-4-dilignol 0%, dehydrodiisoeugenol 50% and eugenol 20%. According to convention (Magnusson and Kligman, 1969), isoeugenol was therefore classified as an extreme skin sensitizer; β-O-4-dilignol as a weak skin sensitizer, dehydrodiisoeugenol as a moderate skin sensitizer and eugenol as a mild sensitizer (Table 1).

Non-RI LLNA

The results are shown in Table 2. Dose dependent increases of the stimulation indices were noted in the animals tested with isoeugenol, dehydrodiisoeugenol and eugenol, and stimulation indices for 3%, 10% and 30% isoeugenol were 1.52 ± 0.49, 2.43 ± 0.45 and 6.73 ± 0.88, respectively. Stimulation indices for 3%, 10% and 30% β-O-4-dilignol were 1.02 ± 0.27, 1.19 ± 0.30 and 1.05 ± 0.20, respectively. Stimulation indices for 3%,

Table 1. Results of the guinea pig maximization test for isoeugenol, isoeugenol dimers and eugenol

Chemical name	Sensitization rate (%)	Grade ^a	Classification ^a
Isoeugenol	100	V	Extreme
β-O-4-Dilignol	0	I	Weak
Dehydrodiisoeugenol	50	III	Moderate
Eugenol	20	II	Mild

^a Classifications were made according to the criterion of Magnusson and Kligmann (1969).

Table 2. Results of non-radioisotopic local lymph node assay with isoeugenol, isoeugenol dimers and eugenol used in this study

% tested	Isoeugenol		β -O-4-Dilignol		Dehydrodiisoeugenol		Eugenol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0%	1.00	0.10	1.00	0.12	1.00	0.11	1.00	0.10
3%	1.52	0.49	1.02	0.27	1.95	0.42	0.75	0.13
10%	2.43	0.45	1.19	0.30	3.09	0.31 ^a	1.46	0.20
30%	6.73	0.88 ^a	1.05	0.20	5.37	0.50 ^a	3.83	0.68 ^a
EC3 (%)	12.7		>30		9.4		22.8	

Results represent mean values and standard errors in four mice

The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group.

^a Significantly different from the concurrent vehicle control (0%) at $P < 0.05$ (Dunnett's test).

10% and 30% dehydrodiisoeugenol were 1.95 ± 0.42 , 3.09 ± 0.31 and 5.37 ± 0.50 , respectively. Stimulation indices for 3%, 10% and 30% eugenol were 0.75 ± 0.13 , 1.46 ± 0.20 and 3.83 ± 0.68 , respectively. Concentrations of 10% or greater exposure of dehydrodiisoeugenol, 30% of isoeugenol and eugenol caused a significant increase in SIs compared with concurrent vehicle controls (Dunnett's multiple comparison tests, $P < 0.05$). Whereas β -O-dilignol did not show any lymph node cell proliferative activity in all concentrations tested. The EC3 values for isoeugenol, dehydrodiisoeugenol and eugenol were calculated using a standard method and found to be 12.7, 9.4 and 22.8, respectively. With regard to the test groups given β -O-dilignol, SI values did not exceed a value of 3 even in the highest dose group, and the EC3 value for this chemical was estimated as >30% for the purposes of comparison.

Discussion

Allergic contact dermatitis is regarded as a serious side effect of chemical products. Eugenol and isoeugenol are components of clove oil and nutmeg oil, and are fragrance chemicals with a spicy scent. They are extensively used in perfumes and deodorants, moreover they are expected to make whitening and antibacterial effects in the cosmetic and dermatological fields (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). However, these chemicals are known to be human contact sensitizers. Isoeugenol is a much stronger sensitizer than eugenol and classified as a moderate allergen, human class 2 (Basketter *et al.*, 2000). Meanwhile eugenol is classified as a weak allergen, human class 3. Isoeugenol has been reported to induce positive responses in the standard LLNA, other murine predictive tests and guinea pig prediction tests (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). The decreased sensitization potency of eugenol by dimerization with the addition of a hydroxyl group was previously reported (Takeyoshi *et al.*, 2004). Isoeugenol is an important cosmetic in-

redient similar to eugenol, but isoeugenol is a stronger human sensitizer than eugenol. Therefore the possibility of reducing the sensitization potency of isoeugenol by dimerization was examined as well as a case of eugenol.

The guinea pig maximization test is a conventional skin sensitization test method, and it has been widely used for regulatory purposes. However, this method is not suitable for quantitative sensitization potency prediction because GPMT requires an independent dose selection for intradermal and topical applications. The LLNA is recognized as a reliable sensitization test, and it is currently accepted by many authorities for regulatory purposes. From the point of view of animal welfare, the LLNA is a preferable test method and it is the first choice sensitization test for the REACH program in the EU. In addition, LLNA can give a quantitative endpoint and it would be suitable for sensitization potency prediction. However, the standard LLNA requires the use of radioisotopes for detecting the lymph node cell proliferative response, and it requires specialized test facility and handling procedures. Meanwhile, non-RI LLNA employed in this study is totally based on the standard LLNA protocol except for using BrdU instead of ³H-thymidine, and it can provide sensitizing potency information as well as standard LLNA (Takeyoshi *et al.*, 2004, 2005). So these two types of major skin sensitization tests were conducted.

The present investigations have shown that this chemical also tested positive in the non-RI LLNA and the GPMT. The derivation of EC3 values provides an opportunity to compare these forms of the assay with respect to sensitivity. In one series of investigations, the EC3 values for isoeugenol and eugenol were recorded as values of approximately 1.3% and 13%, respectively (Basketter *et al.*, 2000). In this study using the non-RI LLNA reported here, the EC3 values for isoeugenol and eugenol were calculated as 12.7% and 22.8%, respectively. The EC3 values obtained in the non-RI LLNA for these chemicals were somewhat higher than those in the standard LLNA. However, the EC3 in non-RI LLNA

for isoeugenol was lower than that for eugenol, thus the quantitative relationship of EC3 values are regarded as the same as the standard LLNA, and the values would be a parameter for the relative sensitization potency. The GPMT was also conducted with the same test chemicals. The sensitization rate for isoeugenol and eugenol were 100% and 20%, respectively. Thus isoeugenol was classified as an extreme sensitizer according to the classification criterion of this method. Similarly eugenol was classified as a moderate sensitizer. The results of non-RI LLNA and guinea pig maximization tests for isoeugenol and eugenol were consistent with the results reported previously.

In this study two types of isoeugenol dimer were tested with different bond-structures in addition to their monomer. Dehydrodiisoeugenol, one of the isoeugenol dimers, showed a 50% sensitization rate in the guinea pig maximization test and was classified as a moderate sensitizer. In the non-RI LLNA, this chemical induced dose dependent lymph node cell proliferation, and its EC3 value was estimated as 9.4%. Meanwhile, the other type of isoeugenol dimer, β -O-dilignol, turned out to have a 0% sensitization rate in the guinea pig maximization test, and was classified as a weak sensitizer. This chemical caused no lymph node cell proliferation, and its EC3 value was estimated as >30%.

Dehydrodiisoeugenol is a dimer in which the monomers are covalently bonded each other, so it would not be easily broken down to monomers in the skin. β -O-Dilignol is a dimer in which the monomers are bonded with ether bond. β -O-Dilignol may be broken down easier than dehydrodiisoeugenol. However, β -dilignol exerts less sensitization potency than its monomer, isoeugenol. It shows that the degradation of β -dilignol in the skin is not significant in this study.

In this study, dimerization of isoeugenol also yielded dimers with different sensitization potencies. Isoeugenol and eugenol are both important cosmetic ingredients, so the reduction of sensitization potency achieved by the dimerization may lead to the development of safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and anti-bacterial effects similar to its monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for cosmetic ingredients with a low sensitization risk. This trial may contribute to a novel strategy to develop safer cosmetic ingredients.

In addition, when these two types of isoeugenol dimer were applied to the DEREK prediction system (Lhasa Limited, Leeds, UK), both dimers were predicted as 'PLAUSIBLE'. More data accumulation would be necessary, but the case studies for eugenol and isoeugenol dimers would provide valuable information to improve the prediction accuracy of SAR systems. A reduction of false-positives in SAR systems would contribute to effective screening of the promising cosmetic and pharmaceutical candidate chemicals.

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