Modulation of biotransformation and genotoxicity by eugenol

Cathy Rompelberg

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Modulatie van biotransformatie en genotoxiciteit door eugenol

(met een samenvatting in het Nederlands)

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Voor mijn ouders

Contents

1. General introduction 9
   - Part I. Dietary chemoprevention of cancer. 9
   - Part II. Toxicity profile of eugenol. 19
   - Part III. Outline of the thesis. 37

2. Habitual use of spices in the Netherlands. 39

3. Effects of the naturally occurring alkenylbenzenes eugenol and *trans*-anethole on drug-metabolizing enzymes in rat liver. 49

4. Effect of eugenol on the genotoxicity of established mutagens in the liver. 69

5. Antimutagenicity of eugenol in the rodent bone marrow micronucleus test. 87

6. Effect of eugenol on the mutagenicity of benzo[a]pyrene and the formation of benzo[a]pyrene-DNA adducts in the λ-*lac*Z-transgenic mouse. 99

7. Effect of short-term dietary administration of eugenol in humans. 117

8. Inhibition of rat, mouse and human glutathione S-transferase by eugenol and its oxidation products. 131

9. Summarizing discussion and conclusions 147

Samenvatting voor niet-vakgenoten 161
Curriculum vitae 164
List of publications 165
Dankwoord 166

CHAPTER 1

GENERAL INTRODUCTION

PART I. DIETARY CHEMOPREVENTION OF CANCER

Nutrition and cancer

Besides the necessity of food for the support of life, many healthy and harmful components of food have been known for centuries. In 1981, Doll and Peto estimated that in the USA the proportional contribution of the diet to cancer deaths was about 30% (Doll and Peto, 1981). Indeed, foods contain mutagens and/or carcinogens, some of which occur naturally and others which can be introduced during the preparation of foods for consumption (Pariza *et al.*, 1990; Wakabayashi *et al.*, 1991). In contrast, recent research indicates that the human diet also contains a number of compounds that protect against cancer (Stich, 1991; Birt and Bresnik, 1991; Verhagen *et al.*, 1993). This is in close agreement with epidemiological findings of negative associations between cancer and consumption of fibre-containing foods, fresh fruits, vegetables, vitamins and minerals (Archer, 1988; Birt and Bresnick, 1991; Steinmetz and Potter, 1991a; Steinmetz and Potter, 1991b). Thus, it appears to be possible to reduce or increase our cancer risk by dietary habits. Nowadays, designer foods - processed foods supplemented with food ingredients naturally rich in cancer-preventing substances - are being studied, assessed and developed (Caragay, 1992; Blenford, 1994). In the future, designer foods may become a new generation of foods, which will protect against cancer and possibly other diseases as well (Caragay, 1992).

Chemopreventive agents in food

Cancer chemoprevention can be defined as 'prevention of cancer by the administration of one or more chemical entities, either as individual drugs or as naturally occurring constituents of the diet' (Morse and Stoner, 1993). Two terms frequently used in cancer

chemoprevention are 'antimutagen' and 'anticarcinogen'. The word 'antimutagen' has a long history and is now used for factors that reduce the rates of spontaneous or induced mutagenesis by various modes of action. Kada (1986) made a distinction among categories of antimutagens and introduced the terms 'desmutagen' and 'bioantimutagen'. Kada defined desmutagens as 'factors that act directly on mutagens or their precursors and inactivate them'; they act outside the cell. Bioantimutagens are defined as 'factors that act on repair and replication processes of the damaged DNA resulting in decreases in mutation frequency' and act inside the cell. Crabtree (1947) defined an anticarcinogen as 'any factor which delays or prevents the emergence of malignant characters in any tissue of any species or organism'.

In recent years much attention has been devoted to investigate compounds in foods with antimutagenic and/or anticarcinogenic potential. Chemopreventive agents are found in all categories of foods (Table 1).

Table 1. Categories of foods with the most prominent chemopreventive agents (Stavric, 1994).

Type of food	Chemopreventive agents
Fruits	Vitamins, flavonoids, polyphenolic acids, fibre, carotenes, monoterpenoids (*d*-limonene)
Vegetables	Vitamins, flavonoids, plant phenolics, chlorophyll, fibre, aliphatic sulphides, carotenes, aromatic isothiocyanates, dithiolthiones, phytic acid, calcium
Cereals	Fibre, α-tocopherol, phytic acid, selenium
Meat, fish, eggs, poultry	Conjugated isomers of linoleic acid, vitamins (A, E), selenites
Fat/oil	Fatty acids, vitamin E, tocotrienols
Milk	Fermentation products, calcium, free fatty acids
Nuts, beans, grains	Polyphenolics, fibre, vitamin E, phytic acid, coumarins, proteins
Spices	Coumarins, curcumin, sesaminol
Tea	Plant phenolics, epigallocatechin
Coffee	Polyphenolic acids, diterpene alcohol esters, melanoidins
Wine	Flavonoids
Water	Selenium

There has been a growing awareness that non-nutrient compounds in the diet, such as phenolics, indoles, aromatic isothiocyanates, terpenes and organosulphur compounds, may modulate the consequences of exposure to carcinogens. Currently, in particular the group of minor non-nutritive dietary constituents is under investigation in the search for inhibitory compounds (Wattenberg, 1983; Wattenberg, 1992; Wattenberg, 1993). Both

synthetic and naturally occurring substances may possess cancer-preventive properties. However, the public call for an 'additive-free' and 'natural' diet directs the main interest towards naturally occurring minor non-nutrients. Many naturally occurring minor non-nutrients (e.g., caffeic acid, catechol, quercetin) are thought to owe their anticarcinogenic effects to their antioxidant potential (Bertram and Frank, 1993). Beyond these effects, however, many other biochemical properties may be responsible for a cancer-preventive potential.

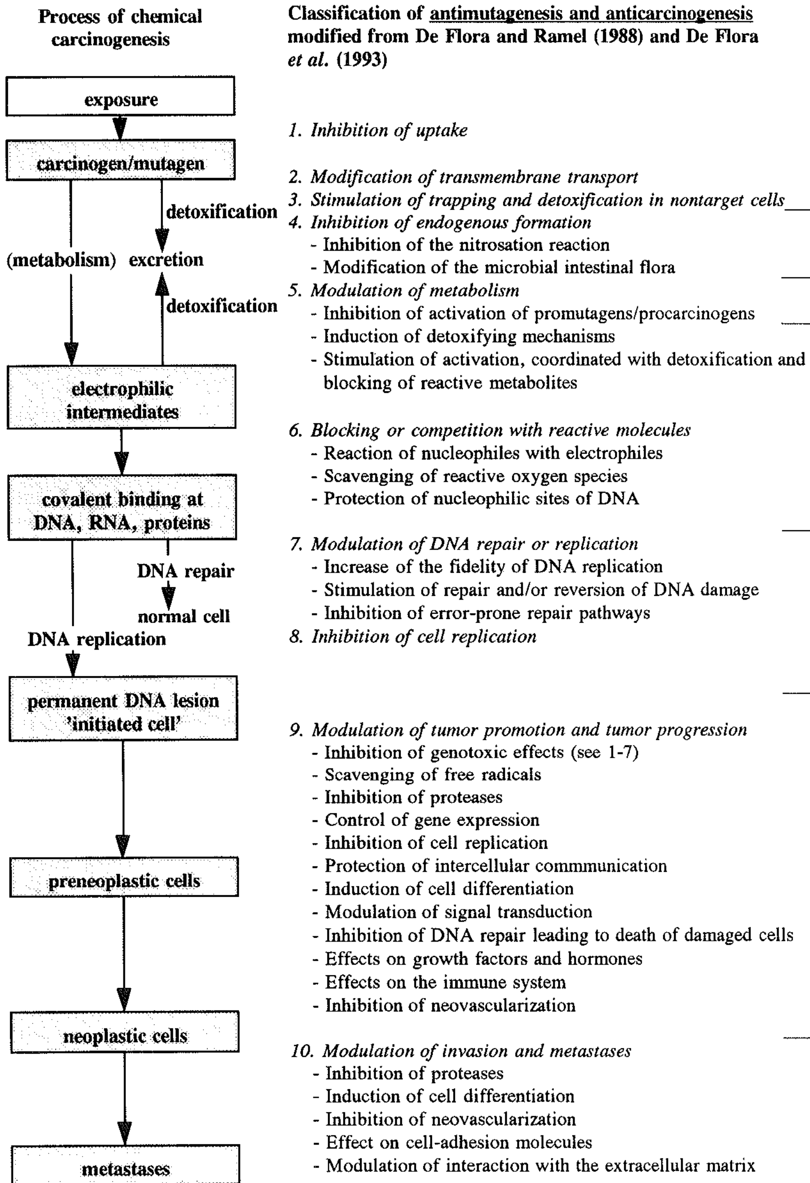
Mechanisms of action

Carcinogenesis is a multistage process. In the simplest model of carcinogenesis the process is assumed to occur in two stages: initiation and promotion/progression. Initiation is the primary event in which cellular DNA undergoes damage which remains unrepaired or is misrepaired. The resulting somatic mutation is reproduced at mitosis, giving rise to a clonal population of 'initiated cells'. 'Initiated cells' do not inevitably lead to a tumour until they have undergone 'promotion', a process that facilitates their further transformation to an invasive state, progression. Compounds that function as promoters are often mitogenic (instead of genotoxic), and may interfere with the expression of genes controlling differentiation, growth and immunomodulation.

The anticarcinogenic and antimutagenic mechanisms of chemopreventive agents are manifold (De Flora and Ramel, 1988; De Flora *et al.*, 1993). The multistage nature of carcinogenesis raises the possibility for intervention at each stage of the process. The beneficial activity of chemopreventors depends on many unrelated factors and conditions. This effect could be the result of a single event or the simultaneous action of several factors acting in concert. For a detailed description of the possible mechanisms of inhibition, the reader is referred to the reviews of De Flora and Ramel (1988), De Flora *et al.* (1993) and Kuroda (1990).

There are many different classifications of the mechanisms of chemopreventive agents, such as those postulated by Hastings *et al.* (1976), Wattenberg (1985), Kada *et al.* (1986), Hartman and Shankel (1990), De Flora *et al.* (1993) and von Borstel and Hennig (1993). The use of these different classifications makes the field of antimutagenesis and anticarcinogenesis unnecessarily complicated. In order to shed light on this, an overview is given of the most commonly used classifications in antimutagenesis and anticarcinogenesis and their mutual connections (Fig. 1). When comparing the classifications of antimutagenesis with those of anticarcinogenesis one should bear in mind that not every antimutagen is an anticarcinogen, and *vice versa*, in analogy to the fact that not every mutagen is a carcinogen, and *vice versa* (Ames, 1989). This is one caveat in the field of antimutagenesis/anticarcinogenesis. Therefore the classifications of antimutagenesis are not necessarily equal to those of anticarcinogenesis.

Fig. 1. General scheme describing the multi-stage process of chemical carcinogenesis and overview of the most commonly used classifications in the field of antimutagenesis/anticarcinogenesis.



Classification of <u>anticarcinogenesis</u> according to Wattenberg (1985)	Classification of <u>antimutagenesis</u> according to Kada *et al.* (1986)
INHIBITORS PREVENTING FORMATION OF CARCINOGENS	DESMUTAGENS[1] (act directly on mutagens or their precursors and inactivate them)
BLOCKING AGENTS (prevent carcinogenic agents from reaching or reacting with critical target sites in the tissues)	
	BIOANTIMUTAGENS[2] (act on repair and replication processes of the damaged DNA resulting in decreases in mutant frequency)
SUPPRESSING AGENTS (suppress the expression of neoplasia in cells previously exposed to doses of a carcinogenic agent that will cause cancer)	

[1] Synonyms: 'countermutagen' (Hastings *et al.*, 1976), 'interceptor' (Hartman and Shankel, 1990)
[2] Synonym: 'fidelogen' (von Borstel and Hennig, 1993)

The choice of a classification mentioned in Figure 1 is merely dependent on the test system used (see next section): with short-term *in vitro* genotoxicity tests only antimutagenesis can be studied while with long-term *in vivo* studies in experimental animals, anticarcinogenesis can be studied. Therefore, we propose that a classification should preferentially be based on referral to the mechanism parallel to the process of chemical carcinogenesis. Based on the stage of the chemical carcinogenesis process with which the compound with cancer-preventive properties intervenes, one obtains insight into its mechanism of action. Finally, by having knowledge of the mechanism underlying antimutagenicity/anticarcinogenicity, one can decide whether a compound is suitable for cancer prevention in the general population or rather for cancer therapy; for example, a compound that prevents the formation of electrophilic intermediates is suitable for application in the general population, while a compound that prevents metastases is suitable for cancer therapy.

Tiered approach for studying (anti)mutagens/(anti)carcinogens

Genotoxicity of a compound is generally tested in a tiered approach: first, short-term *in vitro* tests with prokaryotic or eukaryotic cell systems are performed, such as the Ames test (OECD, 1983a and 1994a; Gatehouse *et al.*, 1994), followed by short-term *in vivo* tests in experimental animals, such as the bone marrow micronucleus test (OECD, 1983b and 1994b; Hayashi *et al.*, 1994). Depending on the results of the short-term genotoxicity tests, a long-term *in vivo* study in experimental animals (OECD, 1981) may be performed, in which the carcinogenic potential of a compound is established by life-time exposure of experimental animals to various dose levels of the test compound up to some level of toxicity (Fig. 2). Occasionally, also epidemiological data and/or data from short-term human biomarker studies are available that can be used in addition to or instead of experimental data with animals (Fig. 2). A biomarker is defined as a parameter, at the biochemical, physiological, enzymatic or cellular level, that reflects some phase between external exposure and eventual effect (disease), and includes factors that may modify transition states between those phases (individual susceptibility, nutrition) (Verhagen *et al.*, 1993).

All these experimental test systems for genotoxicity *in vitro* and *in vivo* and carcinogenicity *in vivo* can equally well be applied to determine the antigenotoxicity or anticarcinogenicity of compounds, by studying the effect of the compound on the response to established genotoxic or carcinogenic agents (Fig. 2). Ferguson (1994) states that it is important for an established antigenotoxic response *in vitro* to be verified *in vivo*. However, one should take into account that if no antigenotoxic potential *in vivo* is evident, a classification as antigenotoxic substance is no longer appropriate (Verhagen and Feron, 1994). This is a second caveat to take into account in determining the antigeno-

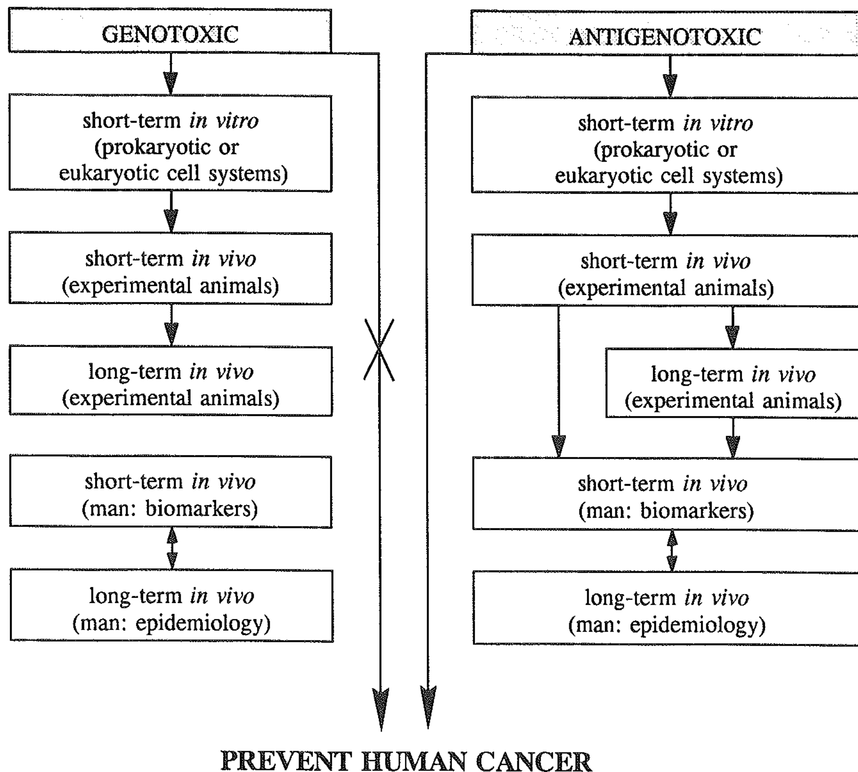


Fig. 2. Tiered approach for studying genotoxicity and antigenotoxicity.

toxic potential of a compound. A third caveat is that dose levels at the target tissue must be sufficiently high to reach the 'lowest beneficial effective level' (LBEL). Exposure to putative antigenotoxic compounds below the LBEL remains necessarily without effect (Verhagen and Feron, 1994).

Because tumour formation is an *in vivo* event, an anticarcinogenic potential can only be assessed in *in vivo* test systems (Verhagen and Feron, 1994). Beyond these experimental studies, the most valuable data on cancer-preventing effects of compounds in the human diet come from human studies. These data can be obtained in the same two ways

as described above: by means of epidemiological studies and by experimental biomarker research in human subjects (Fig. 2). For the appropriate application of biomarkers it is required that one has a profound insight into ethical and practical aspects of studies with human subjects, into the underlying biological mechanisms of antigenotoxicity or anticarcinogenicity as well as into intra- and interindividual variation of the selected biomarkers. If these requirements are met, there are good possibilities for the application of biomarkers in well-chosen study designs.

A fourth caveat in studying antimutagenesis/anticarcinogenesis is that one has to be aware of possible adverse effects of inhibitors. The fact that a compound exerts an inhibitory effect against carcinogenesis in an experimental system does not necessarily mean that it will be beneficial to the host. Some inhibitors have more than one biological action, so that the same substance may prove a hazard to the host in addition to providing some potentially protective effects. Thus, inhibitors must be studied fully for adverse effects in order to evaluate their overall implications for the host's well-being (Wattenberg, 1983). In case of dietary antigenotoxic/anticarcinogenic compounds, a beneficial effect is only valuable in the absence of toxicity. In practice this means that the beneficial effects should be evident at lower dose levels than where toxicity is expected (Verhagen and Feron, 1994).

Conclusions

In the field of antimutagenesis/anticarcinogenesis there are many different classifications for chemopreventive compounds. A classification should preferentially be based on referral to the mechanism parallel to the process of chemical carcinogenesis. In studying the chemopreventive potential of compounds in a tiered approach one should be aware of various caveats: 1) (anti)carcinogens are not (anti)mutagens and *vice versa*, 2) an antigenotoxic potential achieved *in vitro* should be verified *in vivo*, 3) dose levels at the target tissue must be sufficiently high to reach the LBEL, and 4) a beneficial effect is only valuable in the absence of toxicity.

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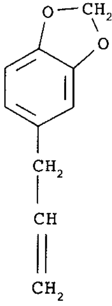
PART II. TOXICITY PROFILE OF EUGENOL

Introduction

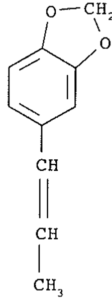
Alkenylbenzenes are an important group of naturally occurring flavour constituents, which are found in a wide range of herb and spice oils. Examples of some naturally occurring alkenylbenzenes are shown in Figure 1. Interest in this class of compounds arises from the finding in the early 1960s that safrole is a hepatocarcinogen in rodents (Homburger *et al.*, 1961), which led to its withdrawal as non-specific food additive in the USA. Subsequently, a number of other congeners have been shown to be rodent carcinogens, including estragole, methyleugenol, α-asarone and ß-asarone. In contrast, eugenol, *trans*-anethole, allylbenzene, isosafrole and myristicin show little or no genotoxicity or carcinogenicity (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Miller and Miller, 1983; Wiseman *et al.*, 1987).

Alkenylbenzenes can be divided into two types, derivatives of allylbenzene and those of propenylbenzene. The mechanism of genotoxicity of allylbenzenes is believed to involve benzylic hydroxylation followed by *O*-sulphation of the alcoholic metabolite and heterolytic cleavage of the *O*-sulphate moiety to form an electrophilic carbonium ion reactive toward a variety of nucleophilic sites in DNA (Fig. 2) (Boberg *et al.*, 1983; Caldwell *et al.*, 1990). Similarly, the ω-hydroxylation and subsequent sulphation of propenylbenzenes could also lead to formation of carbonium ions (Fig. 2).

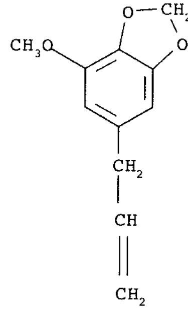
In this thesis the main compound of interest is the naturally occurring alkenylbenzene eugenol (4-allyl-2-methoxyphenol). In short, the identity, the physical and chemical properties, the occurrence, uses and toxicological effects of this compound will be discussed.



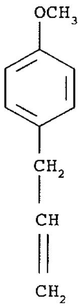
safrole



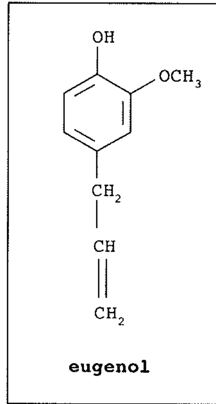
isosafrole



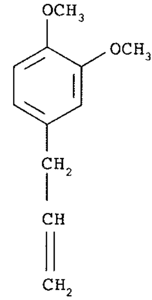
myristicin



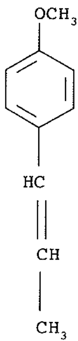
estragole



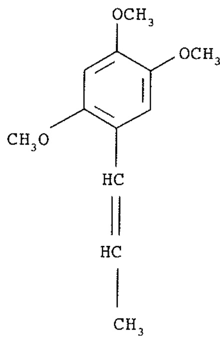
eugenol



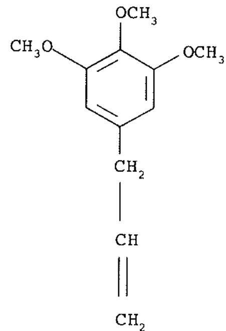
methyleugenol



trans-**anethole**



ß-asarone



elemicin

Fig. 1. Chemical structures of some alkenylbenzenes.

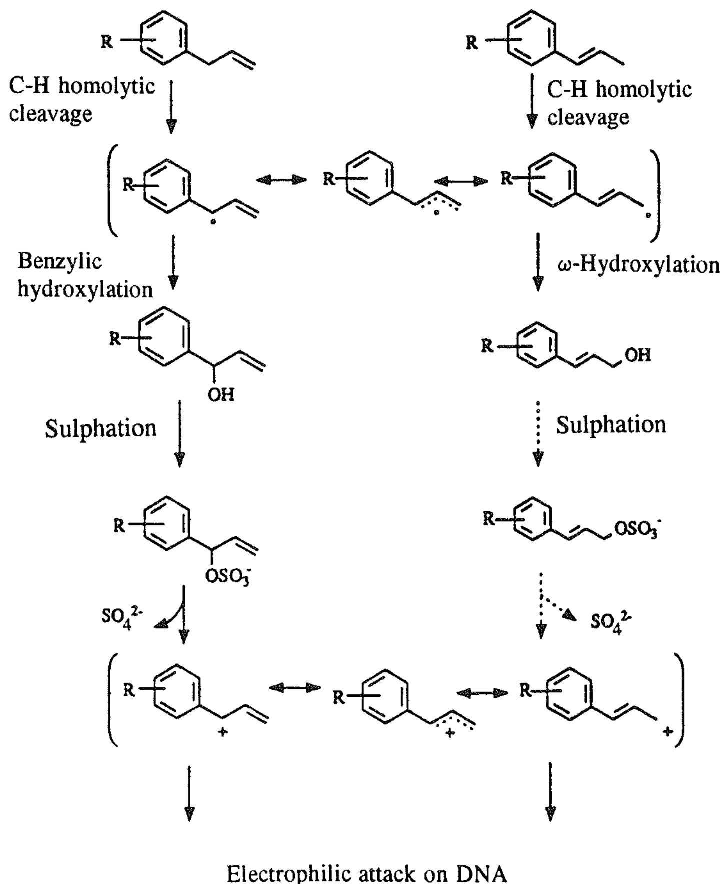


Fig. 2. Postulated mechanisms of metabolic activation of allylbenzenes (left) and propenylbenzenes (right) leading to their genotoxicity. Dotted arrows indicate hypothetical routes (Tsai *et al.*, 1994).

Identity

Molecular formula:	$C_{10}H_{12}O_2$
CAS Reg. No.:	97-53-0
Synonyms:	phenol, 2-methoxy-4-(2-propenyl); 4-allyl-2-methoxyphenol; caryophyllic acid; eugenic acid; 2-methoxy-1-hydroxy-4-allylbenzene; allylguaiacol

Physical and chemical properties

Appearance:	Colourless or pale yellow liquid with an odour of cloves and a spicy, pungent taste
Molecular weight:	164.2
Boiling point:	255°C
Melting point:	-9.2 to -9.1°C
Solubility:	Practically insoluble in water: limited solubility in 70% aqueous ethanol (1 ml in 2 ml); soluble in glacial acetic acid and aqueous alkali; miscible with chloroform, diethyl ether and oils
Volatility:	Vapour pressure, 10 mmHg at 123°C
Stability:	Flash-point, ca. 104°C; darkens and thickens on exposure to air
Reactivity:	Rearranges to isoeugenol when treated with strong alkali
Conversion factor:	1 ppm = 6.71 mg/m^3 at 760 mmHg and 25°C

Natural occurrence

Eugenol is widely distributed in the plant kingdom, where it is mainly found as a component of essential oils. Typical concentrations of eugenol in various plants and derived oils are summarized in Table 1. Furthermore, the Flavor and Extract Manufacturers' Association (FEMA) of the United States (1978) has reported the occurrence of eugenol, without specific concentrations, in the following food sources: cocoa, dried mushrooms, nutmeg, yellow passion fruit, black pepper, peppermint and tomatoes.

Uses

Eugenol, the main component of clove oil, is principally used as a fragrance and flavouring agent, as an insect attractant, as a chemical intermediate, and as an analgesic in dental materials and non-description drug products. The placement of a piece of clove, or clove oil, into carious lesions was common usage in ancient Chinese and Hindu medicine (Molnar, 1942). The dental materials that contain eugenol (e.g. dental cements, impression pastes and surgical pastes) are mostly combinations of zinc oxide and eugenol in varying ratios (Paffenbarger and Rupp, 1979). They are reported to be widely used in

dentistry as temporary filling materials, cavity liners for pulp protection, capping materials, temporary cementation of fixed protheses, impression materials and major ingredients of endodontic sealers (Miller *et al.*, 1978). In addition, eugenol has been used in dentistry for desinfecting root canals (US Food and Drug Administration, 1979).

Eugenol is also a component of clove cigarettes (kreteks), which contain 30-40% crushed clove leaves and 60-70% tobacco leaves (Council on Scientific Affairs, 1988).

Oil of cloves (which is primarily eugenol) has been used as a fragrance raw material since the nineteenth century. Clove-bud oil is used as a flavouring agent in pharmaceuticals, baked goods, sweets, mouthwashes and chewing gum (Rogers, 1981). Clove and its derivatives (oil, buds, leaves, etc, including eugenol) are permitted in the USA for use as flavouring agents and adjuvants (US Food and Drug Administration, 1980).

Table 1. Concentration of eugenol in various plants and derived oils (IARC, 1985).

Plant or derived oil	Concentration (g/kg)
Alfalfa (*Medicago sativa*) oil	7
Basil (*Ocimum gratissimum*)	
light oils	542-639
heavy oils	828-940
Basil (*Ocimum suave* Wild) oils	715
Cinnamon (*Cinnamomum cassia*) bark oil	120
Cinnamon (*Cinnamomum zeylanicum*)	
leaf oil	945
bark oil	420
Clove (*Eugenia caryophyllata*) oil	896-908
buds	918-921
leaves	785-824
Fennel (*Foeniculum vulgare* Miller)	
root essential oil	2
seedling essential oil	3
Jasmine (*Jasminium grandiflorum* L.)	
oil originating from:	
France	11
Italy	8
Algeria	35
Sweet basil (*Ocimum basilicum* L.) essential oil	40-279

Metabolism and pharmacokinetics

In vitro/animal experiments

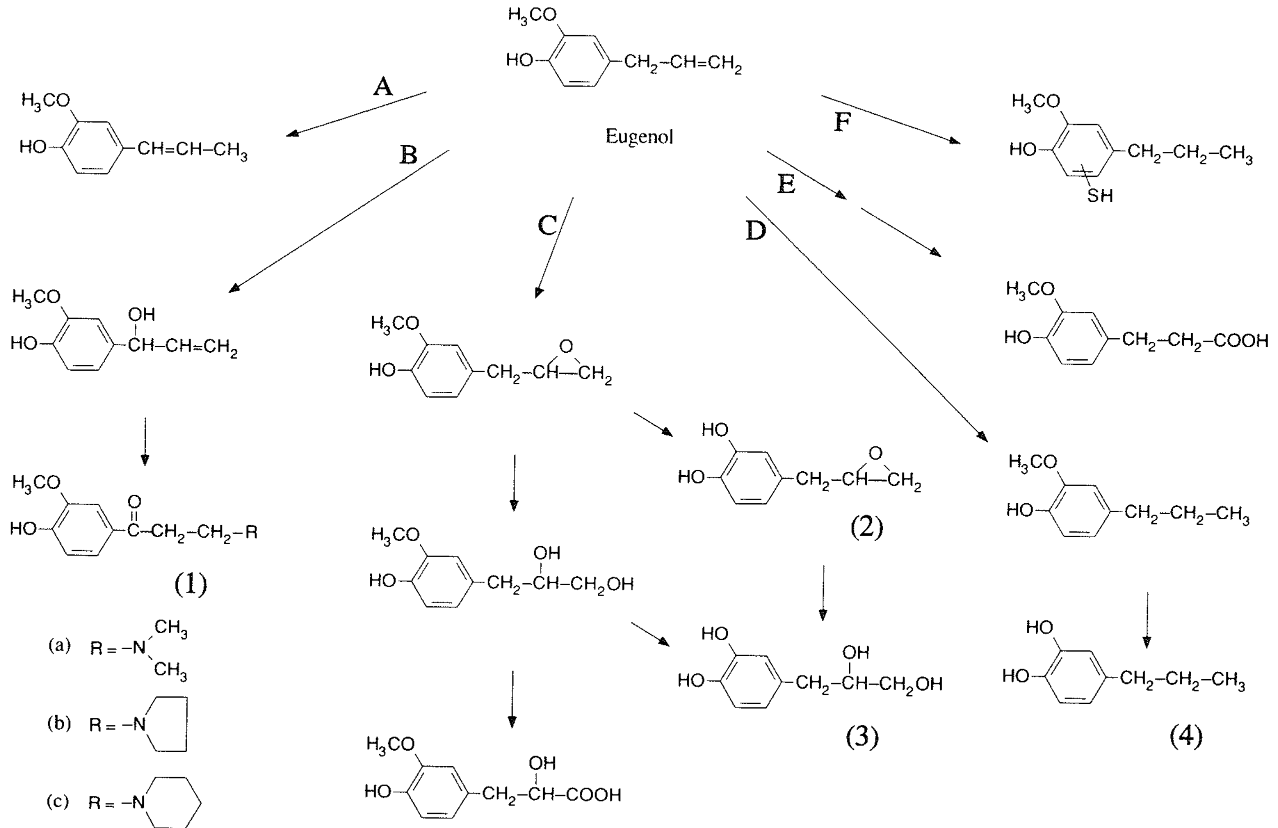
The presence of a free hydroxyl group is an important determinant in the metabolism of eugenol. Caldwell *et al.* (1985) and Sutton *et al.* (1985) noted that benzylic hydroxylation (Fig. 3, pathway B) which may lead to genotoxicity is less extensive with eugenol than with other allylbenzene derivatives. Instead, eugenol is excreted extensively and directly via conjugation of the free hydroxyl group with sulphate or glucuronic acid, with the formation rate of the two conjugates altering with dose (Sutton *et al.*, 1985). In rats and mice the conjugation pattern shifts away from sulphate at low doses toward glucuronic acid at high doses. Other routes of metabolism in rodents include the epoxide-diol pathway (Fig. 3, pathway C) (Delaforge *et al.*, 1980) and reduction of the double bond of the allylic side-chain (Fig. 3, pathway D) (Caldwell *et al.*, 1990).

Eugenol is metabolized via a cytochrome P450-catalysed process to a reactive quinone methide intermediate that is capable of alkylating cellular proteins and thiols (Fig. 4) (Bolton *et al.*, 1995; Thompson *et al.*, 1992; Thompson *et al.*, 1995). There is an accumulating body of evidence that eugenol is also oxidized to a quinone methide by peroxidase (Thompson *et al.*, 1989a,b) (Fig. 4). In situations where protective levels are depleted or compromised, toxicity can result as a consequence of alkylation of critical cellular proteins (Bolton *et al.*, 1995; Thompson *et al.*, 1992; Thompson *et al.*, 1995).

Observations in man

The most detailed study of eugenol metabolism in man was reported by Fischer *et al.* (1990) who administered eugenol (150 mg) orally to human subjects and found that it was rapidly metabolized. Urinary excretion of metabolites was nearly complete in 24 h and 95% of the dose was recovered. Unchanged eugenol accounted for <0.1% of the dose; half of the dose was excreted as glucuronide and sulphate conjugates of eugenol, mainly during the first few hours. Sutton showed that the human metabolism of a low dose of eugenol (0.01 mg/kg b.w.) involves principally glucuronidation (50% of dose) with a further 20% as the sulphate conjugate (Sutton, 1986). All of the compounds shown in Figure 3, except the aminopropiophenone metabolites (1 a,b,c) and the *O*-demethylated derivatives (2, 3 and 4), were identified by Fischer *et al.* (1990). Major metabolic routes are the diol-epoxide pathway (metabolites formed via this pathway account for 13% of the dose) (Fig. 3, pathway C) and formation of a thiophenol (11% of the dose) (Fig. 3, pathway F).

Fig. 3. Metabolism of eugenol (Scheline, 1991). See text for description of pathways A-F.



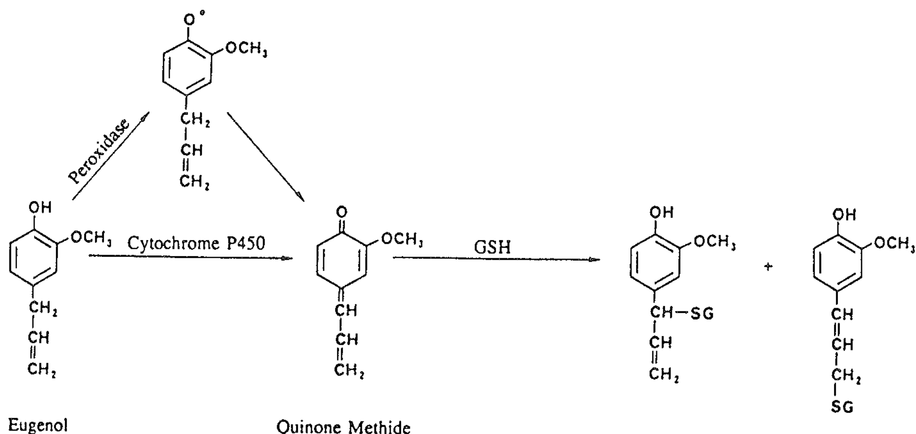


Fig. 4. Oxidation of eugenol to a quinone methide by peroxidase and cytochrome P-450. Glutathione (GSH) adds to multiple sites on the vinylic quinone methide as shown (Thompson *et al.*, 1992).

Effects

In vitro/animal experiments

Irritation and sensitization

Eugenol can be an irritant to lungs (La Voie *et al.*, 1986). In guinea pigs, the skin sensitization potential of eugenol has been predicted to be 20% (Itoh, 1982). Eugenol exerts a low irritating effect on mucous membranes. A 5% emulsion of eugenol administered on a dog's tongue for five minutes produced erythema, ulcers and inflammatory infiltration (Lilly *et al.*, 1972).

Acute toxicity

The acute oral LD_{50} for eugenol has been reported to be 2680 mg/kg in rats (Taylor *et al.*, 1964), 3000 mg/kg in mice (Jenner *et al.*, 1964) and 2130 mg/kg in guinea pigs (Jenner *et al.*, 1964).

The acute mammalian toxicity is associated with a drop in body temperature, muscle weakness, loss of righting reflex, cardiovascular and respiratory effects, and tissue irritation (Dallmeir and Carlini, 1981; Lauber and Hollander, 1950; Sticht and Smith, 1971; Sober, 1950; La Voie *et al.*, 1986).

Subacute and subchronic toxicity

In rats orally treated with doses increasing from 1400 to 4000 mg/kg eugenol for 34 days, a slight liver enlargement with yellow discolouration was seen. Moderately severe hyperplasia and hyperkeratosis associated with focal ulceration were seen in the forestomach (Hagan *et al.*, 1965).

In a 91-day study, rats receiving ≤0.6% eugenol in the diet showed no adverse effects. Administration of 1.2% eugenol in the diet caused weight loss in male rats. No chemically related gross or histopathological effects were observed (NTP, 1983).

Carcinogenicity

The National Toxicology Program investigated eugenol in a 2-year feeding study in rats and mice. Eugenol was given in the diets to female and male rats (0, 0.6 or 1.25%) and female and male mice (0, 0.3 or 0.6%). Under these experimental conditions there was no evidence for carcinogenicity for the rats. For mice there was equivocal evidence for carcinogenicity. In male mice, the low-dose animals (but not the high-dose animals) had increased incidences of both hepatocellular carcinomas and hepatocellular adenomas. In female mice, there was no single liver tumour type with a significantly increased incidence. When the incidences of female mice with hepatocellular adenoma or carcinoma were combined, there was a dose-related positive trend and the incidence of liver neoplasms in high-dose animals was higher than in controls (NTP, 1983). This led to the conclusion that there is limited evidence for carcinogenicity of eugenol in laboratory animals (NTP, 1983). However, this NTP study has been criticized since the increase in tumours at the low dose in male mice might have been due to local room effects rather than to eugenol treatment (Young, 1987).

In female mice fed a diet containing 0.5% eugenol for 12 months followed by a control diet for 6 months no hepatomas were detected (Miller *et al.*, 1983).

Genotoxicity

The results of the genotoxicity studies with eugenol *in vitro* and *in vivo* are summarized in Table 2 and Table 3, respectively. There is only limited evidence for genotoxicity at high dose levels.

Reproduction toxicity

No data are available.

Immunotoxicity

In mice treated orally with different concentrations of eugenol (0.05-10 μmol/kg b.w.), dose-dependent suppressive and enhancing effects of eugenol on the immune response (i.e. the specific antibody response generated to sheep red blood cells antigen and natural killer activity) were found (Vishteh *et al.*, 1986).

Table 2. Genotoxicity of eugenol *in vitro*.

End-point	Test system	Concentration (treatment time)	Result +act	Result -act	Reference
Primary DNA damage	unscheduled DNA synthesis assay (rat hepatocytes)	1-500 μM (16 h)	-	n.a.	Howes *et al.*, 1990
Gene mutation	*Salmonella typhimurium* (TA1530, TA1531, TA1532, TA1964)	6-30 μmol/plate	n.d.	-	Green and Savage, 1978
	Salmonella typhimurium (TA1530, TA1531, TA1532, TA1964)	0.02 M/plate 0.2 M/plate	- -	n.d. n.d.	Green and Savage, 1978
	Salmonella typhimurium (TA98, TA100, TA1535)	0-2 μmol/plate	-	-	Swanson *et al.*, 1979
	Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA1538)	0.4-3.7 μmol/plate	-	-	Sekizawa and Shibamoto, 1982

	Escherichia Coli WP2 *uvrA trp*⁻	0.4-3.7 μmol/plate	-	-	Sekizawa and Shibamoto, 1982
Sister chromatid exchanges	human lymphocytes	0-0.5 mM (64 h)	n.d.	-	Jansson *et al.*, 1986
	CHO cells	3.3-100 μM (21 h)	n.d.	-	Sasaki *et al.*, 1989
Chromosomal aberration	CHL cells	0.76 mM (48 h)	n.d.	+	Ishidate, 1987
	CHO cells	0.30 mM (3 h)	+	-	Stich *et al.*, 1981a
		2.44 mM (3 h)	n.d.	+	
	CHO cells	0.30-1.22 mM (3 h)	n.d.	-	Stich *et al.*, 1981b

CHL= Chinese hamster lung cells
CHO= Chinese hamster ovary cells
+act= with metabolic activation
-act= without metabolic activation
n.a.= not applicable
n.d.= not done

Table 3. Genotoxicity of eugenol *in vivo*.

End-point	Test system	Species	Dose (treatment)	Killing time(s) (time after last dose)	Result	Reference
DNA adducts (liver)	^{32}P-postlabelling assay	CD-1 mice (♀)	80 mg/kg (1x,ip) 400 mg/kg (1x,ip)	24 h 24 h	- -	Randerath *et al.*, 1984
		$B6C3F_1$ mice (newborn ♂)	0.78 mg over the first 22 days of life (ip)	1,7,21 days	-	Phillips *et al.*, 1984
			0.78 mg over the first 22 days of life (ip)	24 h	-	Phillips, 1990
		Parkes mice (♂)	2 mg (1x,ip) 10 mg (1x,ip)	24 h 24 h	- -	Phillips, 1990
		λ-*LacZ*-transgenic mouse strain 40.6 (Muta™Mouse) (♂)	0.4 % in diet (58 days)	24 h	+	Steenwinkel *et al.*, 1995
Gene mutation (peritoneum)	Host-mediated assay	C3H/HeJ mice (♂)	200 mg/kg (1x,im)	3 h	-	Green and Savage, 1978

Micronuclei (bone marrow)	Bone marrow mutagenicity assay	ddY mice (♂)	100 mg/kg (1x,ip)	18,24,30,48,72 h	-	Hayashi *et al.*, 1984
			200 mg/kg (1x,ip)	18,24,30,48,72 h	-	
			400 mg/kg (1x,ip)	18,24,30,48,72 h	-	
			800 mg/kg (1x,ip)	18,24,30,48,72 h	-	
		Swiss mice (♂)	147.9 mg/kg (2x,ip)	6 h	+	Woolverton *et al.*, 1986
			739.9 mg/kg (2x,ip)	6 h	+	
			14,794.4 mg/kg (2x,po)	6 h	+	
		$B6C3F_1$ mice (♂)	150 mg/kg (3x,ip)	48 h	-	Shelby *et al.*, 1993
			300 mg/kg (3x,ip)	48 h	-	
			600 mg/kg (3x,ip)	48 h	-	
		CF1 mice (♂)	100 mg/kg (1x,ip)	30 h	-	Ellahueñe *et al.*,1994
			400 mg/kg (1x,ip)	30 h	+	
			600 mg/kg (1x,ip)	30 h	+	
		CF1 mice (♂)	400 mg/kg (1x,ip)	24,30,48 h	+	Ellahueñe *et al.*,1994
		Sprague-Dawley rats (♀)	168 mg/kg (2x,ip)	6 h	-	Maura *et al.*, 1989
			335 mg/kg (2x,ip)	6 h	-	
			670 mg/kg (2x,ip)	6 h	-	

im = intramuscular
ip = intraperitoneal

Observations in man

Many reports have been published on the high potential of eugenol and of clove-leaf oil (ca. 85% eugenol) for skin sensitization (for a review, see Rothenstein *et al.*, 1983). In human patch tests, the frequency of allergic-type positive reactions to 5% eugenol in cosmetic dermatitis patients was 2.6% (Itoh, 1982). However, at the concentrations present in consumer products, eugenol alone or as part of clove oil has a very low potential either to elicit pre-existing sensitization or to induce hypersensitivity (Rothenstein *et al.*, 1983).

Evaluation/acceptable daily intake

Eugenol and clove oil are generally recognized as safe food additives (GRAS) (FEMA, 1978) and have been approved for food use by the Food and Drug Administration and the Council of Europe. The Joint FAO/WHO Expert Committee on Food Additives established an acceptable daily intake of eugenol for human consumption of 0-2.5 mg/kg of body weight (WHO, 1982). The IARC classified eugenol in group 3, which implies inadequate evidence for carcinogenicity of eugenol to man and limited evidence for carcinogenicity to animals (IARC, 1987).

Antimutagenicity and anticarcinogenicity

Eugenol has several properties that render it a possible candidate antimutagen/anticarcinogen. Firstly, eugenol has been found to be an antioxidative agent that scavenges hydroxyl radicals (Nagababu and Lakshmaiah, 1992). The antioxidant property is essentially due to the phenolic group, which can react with a free radical to form the phenoxyl radical (Rajakumar and Rao, 1993). Secondly, eugenol may also lend protection against endogenous nitrosation because eugenol acts as a nitrosation inhibitor *in vitro* (Nagabhushan *et al.*, 1989). There are indications that the hydroxyl group plays a key role in the anti-nitrosating activity of the molecule (Mulky *et al.*, 1987). Eugenol may be oxidized by the nitrosating agent to a quinone methide, thus depriving the amine of the available nitrite (Shenoy, 1989). A third property of eugenol that makes it a possible candidate antimutagen/anticarcinogen is its induction of detoxifying phase-II biotransformation enzymes (glutathione S-transferase and glucuronyl transferase) *in vivo* in rats and mice (Yokota *et al.*, 1988; Zheng *et al.*, 1992).

Indeed, for eugenol there are indications for protective effects against chemically induced mutagenesis *in vitro*. Eugenol inhibited the mutagenicity of aflatoxin B_1, N-methyl-N'-nitro-N-nitrosoguanidine and dimethylbenzanthracene in the Ames test

(Amonkar *et al.*, 1986; Francis *et al.*, 1989). In addition, microsomes or S9 prepared from rats that received eugenol decreased the mutagenic activity of benzo[a]pyrene in the Ames test in comparison to the microsomes or S9 from untreated rats (Yokota *et al.*, 1986).

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PART III. OUTLINE OF THE THESIS

The purpose of the these studies was to obtain insight into the antigenotoxic potential of herbs and spices. Alkenylbenzenes were chosen as model compounds. Before investigating the antigenotoxic potential of herbs and spices we wished to get insight into the habitual use of herbs and spices in the general population. Therefore, data on the use of herbs and spices collected in two epidemiological studies have been analysed; the results are shown in Chapter 2. In the next chapters investigations were performed to study the antigenotoxic potential of alkenylbenzenes. In the first study (Chapter 3) one important mechanism for antigenotoxicity, i.e. induction of biotransformation enzymes, was studied in rats treated orally with eugenol or *trans*-anethole. In the next chapters the (anti)genotoxic potential of eugenol was studied *in vitro* (Chapter 4) and *in vivo* (Chapter 5 and 6) using different assays. Chapter 7 describes an intervention study with human volunteers, to study the antigenotoxic potential of eugenol and the effect of eugenol on biotransformation enzymes in man. Because differences in effect of eugenol on glutathione S-transferase activities were found in several of the studies performed, the effect of eugenol on glutathione S-transferase activities was studied further *in vitro* with liver cytosol and purified isoenzymes of animals and men (Chapter 8). In Chapter 9 the results presented in this thesis are discussed.

CHAPTER 2

HABITUAL USE OF SPICES IN THE NETHERLANDS

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Abstract

Information on the habitual use of spices (cloves, nutmeg, curry, pepper and garlic) in the Dutch population aged 20-79 was collected by means of an open-ended and a predefined food frequency questionnaire. As reference, repeated 24-h recalls were used. The consumption of clove, nutmeg, curry, pepper and garlic (related to age and gender) and the frequency of use of clove, nutmeg, curry and garlic were estimated. The consumption of spices varied depending on the type of assessment method. On the basis of the defined food frequency questionnaire, clove, nutmeg, curry and garlic were used by at least 60% of the population. The open-ended food frequency questionnaire yielded similar consumption figures for nutmeg and pepper, but the proportion of subjects reporting to consume clove (6%) or curry (13%) was much lower. There were age-related differences in consumption of spices. Elderly subjects (≥ 60 years) more often reported to eat nutmeg and less often reported to use garlic. No gender-related difference in consumption of spices was found in the open-ended or the predefined food frequency questionnaire. Though, for garlic a gender effect was found in the reference method: men more often reported to use garlic than women. For the majority of subjects frequency of consumption of clove, nutmeg, curry, and garlic as measured by the predefined food frequency questionnaire was ≤5 times per month. This was confirmed in the 24-h recalls. Results

obtained with the defined food frequency questionnaire most closely approximated those obtained by repeated 24-h recalls. It is concluded that for determination of habitual spice consumption a predefined food frequency questionnaire, rather than open-ended questions, appears to be the method of choice.

Introduction

In recent years much attention has been devoted to investigations of compounds in foods with antimutagenic and/or anticarcinogenic potential. There has been a growing awareness that non-nutrient compounds in the diet, such as phenols, indoles, aromatic isothiocyanates, terpenes and organosulphur, may modulate the effects of exposure to carcinogens [1]. Currently, in particular the group of minor nonnutritive dietary constituents is under investigation in the search for inhibitory compounds [2,3]. Spices contain a number of minor non-nutritive compounds with a possible cancer-preventive potential. The public health importance of these compounds depends on the strength of their preventive action, and their use (number of consumers, consumption frequency, and amount).

Several studies have been published on the antimutagenic/anticarcinogenic potential of spices [4,5,6,7,8] but the authors were not aware of any other reports on the extent of their use in the general population. Therefore, the present study has been undertaken to get insight into the number of people in the general Dutch population that consume specific spices and in their consumption frequencies.

The data were taken from two studies. The first one was a case-control study on exocrine pancreatic carcinoma in which subjects were asked to report the names of the three most commonly used spices as part of a food frequency questionnaire. The second source was a dietary validation study. The test method in this study was a food frequency questionnaire in which the consumption frequency of clove, nutmeg, curry and garlic was inquired. As a reference method, a 24-h recall repeated twelve times was administered in which the use of these same spices was studied. Thus, information on spice use was derived from three different dietary assessment methods.

Here we report habitual use by age and gender of some of the most commonly eaten spices (clove, nutmeg, curry, pepper and fresh garlic). In addition, we compare the findings of both questionnaire-based methods with the results obtained by the repeated 24-h recall method.

Subjects and methods

Case-control study

During 1984-1988 a population-based case-control study was carried out in the Netherlands by the National Institute of Public Health and Environmental Protection, in collaboration with the International Agency for Research on Cancer, in order to investigate the role of the diet in exocrine pancreatic carcinoma [9]. The geographic regions in the centre of the Netherlands included the province of Utrecht, western parts of Gelderland, and eastern parts of Zuid-Holland. For the present analysis, 343 population controls 40 to 79 years of age were sampled from the municipal population registries, i.e. 162 men (response 72.7%) and 181 women (response 64.5%). An interviewer-administered semi-quantitative food frequency questionnaire was used to assess recent habitual diet. Subjects were asked to report the names of the spices they used the most, the second most, and the third most. The data of the controls were used to calculate the percentage of users of cloves, nutmeg, curry and pepper.

Dietary validation study

In this study, performed to validate a self-administered food frequency questionnaire [10], subjects were recruited from two study populations of ongoing projects. One of the populations consisted of women in the age range of 50-70, who participated in a breast cancer screening programme in the city of Utrecht. The other population took part in the Monitoring Program for Cardiovascular Risk Factors conducted in the cities of Amsterdam, Doetinchem and Maastricht and was aged 20 to 60. Of the subjects invited to participate 25% responded positively. Data collection started in October 1991 and took 13 months. In the first month the food frequency questionnaire was administered. Subjects were asked to report how often they had usually used clove, nutmeg, curry and fresh garlic in the past year. They could indicate their answers in times per day, per week, per month or per year, or as never.

As reference method 24-h recall interviews were performed monthly during the months 2 through 13, according to a standardized protocol. In the interviews on Tuesday to Saturday subjects were asked whether they had used clove, nutmeg, curry and fresh garlic on the previous day. Half of the interviews on Monday concerned the previous Sunday and the other half the previous Saturday. For most of the subjects, the interview days were evenly distributed over all days of the week except Sunday. The number of recalls in which spice use was reported was extrapolated to frequencies per month. For 113 out of 134 subjects (63 men and 50 women) complete spice data were available.

Statistics

Differences in the percentage of users of spices determined by both food frequency methods and the 24-h recall method were tested across gender and age groups using a χ^2 test [11]. P-values below 0.05 were considered to be statistically significant.

Results

In Table 1, the percentages of users of clove, nutmeg, curry and pepper are shown, based on data obtained by the open-ended food frequency questionnaire. In the total population, 6, 73, 13 and 74% reported use of clove, nutmeg, curry and pepper, respecti-

Table 1. Consumption of spices, as estimated by an *open-ended food frequency questionnaire*, among 162 male and 181 female control subjects participating in a population-based case-control study on diet and cancer of the pancreas by age and gender, the Netherlands, 1984-1988.

Spice	Age (year)	Percentage of population that uses spices (n)					
		Men		Women		Total	
clove	40-59	4	(2)	10	(4)	7	(6)
	60-69	10	(5)	1	(1)	5	(6)
	70-79	7	(4)	6	(4)	6	(8)
	total	7	(11)	5	(9)	6	(20)
nutmeg	40-59	61	(31)	68	(27)	64*	(58)
	60-69	72	(36)	86	(59)	80*	(95)
	70-79	74	(45)	75	(54)	74*	(99)
	total	69	(112)	77	(140)	73	(252)
curry	40-59	16	(8)	18	(7)	16	(15)
	60-69	14	(7)	13	(9)	13	(16)
	70-79	12	(7)	11	(8)	11	(15)
	total	14	(22)	13	(24)	13	(46)
pepper	40-59	75	(38)	75	(30)	75	(68)
	60-69	76	(38)	84	(58)	81	(96)
	70-79	71	(43)	65	(47)	68	(90)
	total	74	(119)	75	(135)	74	(254)

*$P<0.05$ ($\chi^2=7.0$)

Table 2. Consumption of spices and consumption frequencies by age and gender as estimated by a *predefined food frequency questionnaire* among 63 male and 50 female subjects participating in a dietary validation study, the Netherlands, 1991-1992.

Spice	Age (year)	Percentage of users (n)	MEN	Percentage of users (n) frequency/month			WOMEN	Percentage of users (n) frequency/month		
			total	$\leq$5	6-10	>10	total	$\leq$5	6-10	>10
clove	20-39	48 (24)*	45 (14)	79 (11)	14 (2)	7 (1)	53 (10)	100 (10)	0	0
	40-59	78 (42)*	81 (26)	92 (24)	0	8 (2)	73 (16)	100 (16)	0	0
	60-70	56 (5) *	-	-	-	-	56 (5)	60 (3)	20 (1)	20 (1)
	total	63 (71)	63 (40)	88 (35)	5 (2)	7 (3)	62 (31)	94 (29)	3 (1)	3 (1)
nutmeg	20-39	74 (37)	74 (23)	78 (18)	9 (2)	13 (3)	74 (14)	86 (12)	0	14 (2)
	40-59	89 (48)	84 (27)	48 (13)	19 (5)	33 (9)	96 (21)	57 (12)	24 (5)	19 (4)
	60-70	78 (7)	-	-	-	-	78 (7)	29 (2)	14 (1)	57 (4)
	total	81 (92)	79 (50)	62 (31)	14 (7)	24 (12)	84 (42)	62 (26)	14 (6)	24 (10)
curry	20-39	66 (33)	68 (21)	90 (19)	0	10 (2)	74 (14)	79 (11)	14 (2)	7 (1)
	40-59	67 (36)	59 (19)	95 (18)	0	5 (1)	77 (17)	88 (15)	12 (2)	0
	60-70	78 (7)	-	-	-	-	78 (7)	86 (6)	0	14 (1)
	total	67 (76)	63 (40)	93 (37)	0	7 (3)	76 (38)	84 (32)	11 (4)	5 (2)
garlic	20-39	74 (37)**	77 (24)	67 (16)	25 (6)	8 (2)	68 (13)	39 (5)	23 (3)	39 (5)
	40-59	72 (39)**	69 (22)	41 (9)	27 (6)	32 (7)	77 (17)	65 (11)	12 (2)	23 (4)
	60-70	33 (3) **	-	-	-	-	33 (3)	67 (2)	33 (1)	0
	total	70 (79)	73 (46)	54 (25)	26 (12)	20 (9)	66 (33)	55 (18)	18 (6)	27 (9)

* $P<0.05$ ($\chi^2=10.1$)
**$P<0.05$ ($\chi^2=6.3$)

Table 3. Consumption of spices and consumption frequencies by age and gender as estimated by *repeated 24-h recall interviews* among 63 male and 50 female subjects participating in a dietary validation study, the Netherlands, 1991-1992.

Spice	Age (year)	Percentage of users (n)	MEN total	MEN Percentage of users (n) frequency/month ≤5	6-10	>10	WOMEN total	WOMEN Percentage of users (n) frequency/month ≤5	6-10	>10
clove	20-39	34 (17)	42 (13)	92 (12)	8 (1)	0	21 (4)	50 (2)	50 (2)	0
	40-59	33 (18)	31 (10)	80 (8)	20 (2)	0	36 (8)	100 (8)	0	0
	60-70	11 (1)	-	-	-	-	11 (1)	100 (1)	0	0
	total	32 (36)	37 (23)	87 (20)	13 (3)	0	26 (13)	85 (11)	15 (2)	0
nutmeg	20-39	60 (30)	65 (20)	90 (18)	5 (1)	5 (1)	53 (10)	60 (6)	20 (2)	20 (2)
	40-59	78 (42)	72 (23)	39 (9)	35 (8)	26 (6)	86 (19)	74 (14)	21 (4)	5 (1)
	60-70	89 (8)	-	-	-	-	89 (8)	25 (2)	50 (4)	25 (2)
	total	71 (80)	68 (43)	63 (27)	21 (9)	16 (7)	74 (37)	59 (22)	27 (10)	14 (5)
curry	20-39	48 (24)	52 (16)	75 (12)	19 (3)	6 (1)	42 (8)	63 (5)	25 (2)	12 (1)
	40-59	46 (25)	44 (14)	79 (11)	21 (3)	0	50 (11)	82 (9)	9 (1)	9 (1)
	60-70	56 (5)	-	-	-	-	56 (5)	40 (2)	60 (3)	0
	total	48 (54)	48 (30)	77 (23)	20 (6)	3 (1)	48 (24)	67 (16)	25 (6)	8 (2)
garlic	20-39	50 (25)*	52 (16)	62 (10)	25 (4)	13 (2)	47 (9)	45 (4)	11 (1)	44 (4)
	40-59	65 (35)*	75 (24)	62 (15)	25 (6)	13 (3)	50 (11)	37 (4)	27 (3)	36 (4)
	60-70	22 (2) *	-	-	-	-	22 (2)	100 (2)	0	0
	total	55 (62)	63 (40)**	62 (25)	25 (10)	13 (5)	44 (22)**	46 (10)	18 (4)	36 (8)

* $P<0.05$ ($\chi^2=6.5$)
** $P<0.05$ ($\chi^2=4.3$)

vely, in their top three of most commonly used spices. For nutmeg, a significant difference was found in the percentage of total users among the different age classes: elderly subjects more often reported to eat nutmeg. Although the reference method did not show a statistically significant difference in consumption of nutmeg by age, the proportion of elderly people (≥ 60 years) reporting to eat nutmeg was also higher. No differences in consumption of spices by gender were found.

Table 2 shows the percentages of users of clove, nutmeg, curry and fresh garlic, subdivided by frequency of use, based on data obtained by the predefined food frequency questionnaire. In total, 63, 81, 67 and 70% reported the use of clove, nutmeg, curry and fresh garlic, respectively. These percentages were higher for all spices than those obtained by 24-h recall interviews: 32, 71, 48 and 55% respectively (Table 3). In both the predefined food frequency questionnaire and the 24-h recalls, most users of clove, nutmeg, curry and garlic reported to use them with a frequency of ≤5 times per month (Table 2 and 3).

In the predefined food frequency questionnaire subjects aged 40 to 59 more often reported to eat clove than younger and older subjects. Elderly subjects (≥ 60 years) less often reported to use garlic. For garlic, but not for clove, this finding was confirmed in the 24-h recall interviews. In the predefined food frequency questionnaire no significant differences in consumption of spices by gender were found. In the 24-h recall method, the percentage of users of garlic was significantly higher for men than for women.

Discussion

Spices contain minor non-nutrients that may have preventive potential against cancer and other diseases. To get insight into the public health importance of these compounds the habitual use of spices in the Dutch population was estimated.

In the open-ended food frequency questionnaire, nutmeg and pepper are the most commonly reported spices. Clove and curry are reported less frequently in the top three of most commonly used spices. This seems to contrast with the results of the predefined food frequency questionnaire in which frequencies of consumption of clove, nutmeg, curry and garlic were all high. The contrast in results of the open-ended and predefined food frequency questionnaire for clove and curry are likely due to differences in the type of questions on spice use. In the open-ended food frequency questionnaire, subjects were asked to report the names of the spices they use the most, the second most and the third most. This means that the non-user category may include subjects that did eat spices, but less frequently than the three most commonly used spices. Consequently, an

underestimation of the percentage of users of less frequently used spices will occur. In the predefined food frequency questionnaire, subjects were asked whether they used a specific spice. Such a predefined question seems to provide more reliable results on specific spice consumption than an open-ended question. However, in a pilot study, an open-ended question can be used to identify the most commonly used spices.

In the 24-h recall method, for all spices the percentage of users was lower than in the predefined food frequency questionnaire. This may be explained by differences in dietary assessment methods. The number of 12 recalls was determined by the within- and between-subject variation in intake of macronutrients and vitamins, and not of spice use. This may imply that the number of repeated recalls is not optimal for an accurate estimation of the use of spices. It is unknown how the low response (25%) in the validation study influences the data of the predefined food frequency questionnaire and the 24-h recalls.

In addition, in all three methods, percentages of users of spices and frequency of consumption may also be underestimated because spices present in ready-to-eat foods were not taken into account. It would probably be very difficult to obtain this information from subjects.

The intake of minor nonnutrients (e.g. spices) may be better measured by using biomarkers of exposure. However, most biomarkers reflect actual consumption rather than habitual dietary intake. In the case of minor non-nutrients the use of biomarkers may present technical difficulties to measure the (very) low concentrations of minor non-nutrients in body fluids. For some minor non-nutrients, biomarkers have been developed [12,13].

The predefined food frequency questionnaire seems to be a good method to estimate the consumption frequencies of spices because the same results were found in the 24-h recalls. It seems likely that in the Netherlands, most users of clove, nutmeg, curry and garlic eat these spices with a frequency of ≤ 5 times per month.

For nutmeg and garlic, differences in consumption across age groups were found. In the open-ended food frequency questionnaire, elderly subjects ($\geq$ 60 years) more often reported to eat nutmeg. In the predefined food frequency questionnaire, subjects younger than 60 more often used garlic. Both effects were confirmed in the reference method. It seems likely that in The Netherlands elderly people more often eat nutmeg, while younger people more often eat garlic. The effect of age on consumption of clove found in the predefined food frequency questionnaire, but not in the reference method, is probably a statistical chance finding.

In the present study there is limited evidence for a gender-related effect on the use of spices. Only in the reference method, men more often reported to use garlic than women.

On the basis of the present results, it may be concluded that predefined questions in a food frequency questionnaire provide more accurate estimations of spice consumption than an open-ended question and appear to be an appropriate method to estimate habitual use of spices. The data of the predefined food frequency questionnaire on use of spices may be used as a crude estimate of the habitual use of spices in the Netherlands.

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treated with eugenol (500 or 1000 mg/kg body weight) or *trans*-anethole (250 mg/kg body weight). It is concluded that both eugenol and *trans*-anethole preferentially induce phase-II biotransformation enzymes in rat liver *in vivo*.

Introduction

Humans are exposed to a large variety of chemical substances from natural sources. In recent years it has become clear that naturally occurring compounds can have important effects on the consequences of exposure to carcinogens. Current scientific interest is directed towards the cancer-preventing potential of naturally occurring substances. Several groups of naturally occurring compounds, including flavonoids, indoles, aromatic isothiocyanates and terpenes, have been shown to possess antimutagenic and anticarcinogenic activities [1,2]. However, especially in spices and herbs numerous other classes of chemicals occur, of which relatively little is known about potential adverse or beneficial effects on carcinogens and mutagens.

Alkenylbenzenes are an important group of naturally occurring food flavourings, comprising eugenol (4-allyl-1-hydroxy-2-methoxybenzene) and *trans*-anethole (1-methoxy-4-propenylbenzene) (Fig. 1). *trans*-Anethole occurs naturally in the volatile oils of a variety of plants, in particular fennel and Chinese star anise, and is used as an aniseed flavouring in sweets, baked goods and (alcoholic) beverages. Eugenol is the main component of oil of cloves and is also present in the essential oils of many other plants, such as cinnamon, basil, and nutmeg.

In 1961 safrole (4-allyl-1,2-methylene-dioxybenzene) was found to be hepatocarcinogenic in rodents [3]. Subsequently, a number of congeners have also been shown to be rodent carcinogens including estragole (4-allyl-1-methoxybenzene) and methyleugenol (4-allyl-1-methyl-2-methoxybenzene) [4,5]. The carcinogenicity of these compounds is dependent on their metabolism to electrophilic intermediates, in particular the 1'-hydroxy-metabolites [6].

With respect to eugenol, a carcinogenicity study was reported by the National Toxicology Program [7]. In mice, but not in rats, there was equivocal evidence of carcinogenicity since eugenol caused increased incidences of liver tumours in females fed 0.3 or 0.6% eugenol in the diet for 103 weeks; in males the increase was significant only for those treated with the lower dose of eugenol. This led to the conclusion that there is limited evidence for carcinogenicity of eugenol in laboratory animals. However, this NTP study has been criticized since the increase in tumours at the low dose in male mice might have been due to local room effects rather than to eugenol treatment [8].

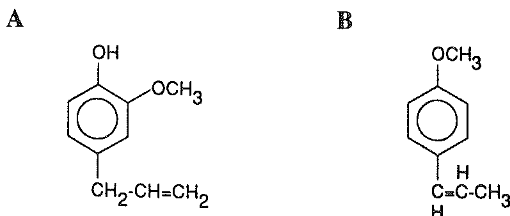

Fig. 1. The chemical structures of (a) eugenol and (b) *trans*-anethole.

Miller *et al.* [5] found that eugenol and *trans*-anethole did not induce hepatic tumours in female mice given 0.5% eugenol or *trans*-anethole in the diet for 12 months. The compounds were also not hepatocarcinogenic when administered i.p. to male mice during the preweaning period at total doses of up to 9.45 μmol/mouse [5].

In a lifetime toxicity/carcinogenicity study in rats *trans*-anethole caused a small but significant increase in the incidence of hepatocellular carcinoma in female rats administered 1% *trans*-anethole in the diet for 121 weeks [9]. The authors emphasized that the low incidence of hepatocarcinomas was restricted to a single species and sex and to the highest dose tested. It has been suggested that the effect of *trans*-anethole on the liver may be the consequence of enzyme induction of mainly cytochrome P-450 isoenzymes and enhanced cell proliferation [10,11,12].

In short-term genotoxicity assays, eugenol and *trans*-anethole were generally negative, whereas safrole, estragole and methyleugenol exhibited genotoxic activity [13,14,15,16]. Therefore, the conclusion that eugenol and *trans*-anethole may be considered non-genotoxic carcinogens seems justifiable, since their carcinogenicity only becomes effective at very high dose levels. As a consequence other, potentially beneficial, effects at lower doses of eugenol and *trans*-anethole may be more relevant to humans.

Nowadays, many naturally occurring dietary constituents are being studied for antimutagenic or anticarcinogenic activity [17]. As concerns one of the mechanisms underlying antimutagenicity/anticarcinogenicity, there appears to be a relationship between the ability of certain xenobiotics (e.g. phenolic antioxidants or azo-dyes) to act as anticarcinogens, and their ability to induce a set of enzymes involved in carcinogen detoxification and excretion [18]. These include the non-oxidative enzymes UDP-glucuronosyl transferase (GT), glutathione S-transferase (GST), and DT-diaphorase (DTD). Therefore, as a first step to determine the potential cancer modulatory effects of eugenol and *trans*-anethole, the effects of oral administration of eugenol and *trans*-anethole on drug-metabolizing enzymes in rat liver were examined.

Materials and methods

Chemicals

All chemicals were of analytical reagent grade. Eugenol (purity 99%) was obtained from Janssen Chimica (Tilburg, The Netherlands) and *trans*-anethole (purity > 98%) from Merck (Schuchardt, Germany).

Animals and treatment

Male Wistar rats (Crl:(WI)WU BR), 8 weeks old, were obtained from Charles River Wiga (Sulzfeld, Germany). They were housed in groups of three in suspended hanging type stainless-steel wire-mesh-bottomed cages, in an air-controlled room (21 ± 1°C) with a relative humidity of 70 ± 10% and a light/dark cycle of 12 hr. All rats were fed an open-formula basal diet for rodents that is commonly used at the TNO Toxicology and Nutrition Institute. Food and water were available *ad libitum.*

Eugenol and *trans*-anethole were administered, dissolved in corn oil, by gavage daily for 10 days. For *trans*-anethole the doses used were 125, 250 and 500 mg/kg body weight and for eugenol they were 250, 500 and 1000 mg/kg body weight. The dosing regimens for eugenol and *trans*-anethole were chosen on the basis of published LD_{50} values. The LD_{50} (oral) of *trans*-anethole is 2090 mg/kg (95% confidence limits 1420-3070) and the LD_{50} (oral) of eugenol is 2680 mg/kg (95% confidence limits 2420-2970) [19]. In the present study the highest doses of *trans*-anethole and eugenol used correspond to approximately 25-35% of the reported LD_{50} values. The control group was given corn oil alone. In all instances doses were administered in a volume of 10 ml/kg body weight. 24 hr after the last dose the rats were killed under ether anaesthesia and blood was collected from the aorta into tubes containing EDTA or heparin. Livers were perfused *in situ* by way of the portal vein with 1% heparin in phosphate buffered saline (0.1 M, pH 7.2) untill yellow, and then snap-frozen in liquid nitrogen and stored at -80°C.

In EDTA-blood samples various haematological parameters were determined: numbers of erythrocytes, leucocytes and platelets, haemoglobin content and haematocrit. Aliquots of 2 ml heparin-blood were centrifuged for 20 min at 1400 x *g*; plasma was stored at -80°C.

Preparation of microsomes and cytosols

Frozen livers were thawed on ice and homogenized in four volumes of ice-cold 1.15% KCl using a Potter-Elvehjem glass-teflon homogenizer. The cell debris, nuclei and mitochondria were removed by centrifugation at 10.000 x *g* for 2 x 20 min at 0-4°C in a

Beckman L8-70 ultracentrifuge. The supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant representing the cytosolic fraction was seperated and the pellet containing the microsomal fraction was resuspended in ice-cold sodium phosphate buffer (pH 7.4, 0.1 mM-EDTA). Aliquots of 1 ml cytosolic and microsomal fractions were quickly frozen in liquid nitrogen and stored at -80°C until use.

Enzyme assays

Cytosolic and microsomal protein content was quantified by the method of Lowry *et al.* [20] using bovine serum albumin as a standard. Total microsomal cytochrome P-450 content was determined according to the method of Rutten *et al.* [21].

Microsomal dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) was assayed by the fluorimetric method of Rutten *et al.* [22]. Less than 70 μg microsomal protein was used in an incubation mixture of 0.1 M-Tris-HCl buffer (pH 7.8) and NADPH-regenerating system in a final volume of 320 μl. Substrate concentrations of 7-ethoxyresorufin and 7-pentoxyresorufin were 7 and 10 μM, respectively.

Microsomal GT activity was determined using 4-chlorophenol and 4-hydroxybiphenyl as substrates as described by Mulder and van Doorn [23]. GST activity was determined using 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* [24].

DTD activity was assayed by the method of Ernster [25] with some modifications. Incubations were carried out in 320 μl Tris buffer (28 mM, pH 7.5) containing 660 μM-NADH, 6.5 μM-FAD, 0.22% Tween-20, 0.08% bovine serum albumin (BSA), 1-2 μg cytosolic protein and 65 μM-2,6-dichlorophenolindophenol as the substrate. The decrease in absorbance was followed at 600 nm for 3 min with and without 10 μM dicumarol.

The methods for determination of EROD, PROD, GT, GST and DTD activities were performed on a Cobas-Bio centrifugal analyser [26].

Levels of L-aspartate aminotransferase (ASAT) and L-alanine aminotransferase (ALAT) were assessed in plasma using a Cobas-Bio centrifugal analyser as described by the Scandinavian Society for Clinical Chemistry and Clinical Physiology [27]. Blood cells were counted with electric resistance detection on a Sysmex K-1000 haematology analyser.

HPLC of GST subunits

Isolation of GST and separation of GST subunits were performed as described by Bogaards *et al.* [28]. In short, liver cytosol (850 μl) was applied to a *S*-hexylglutathione affinity chromatography column. From the *S*-hexylglutathione eluate, 75 μl was injected onto the HPLC column. Separation of the GST subunits was performed on a Vydac 201 TP 5 (200 x 3 mm i.d.) chromatography column (Chrompack, The Netherlands) using a

gradient of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The gradient consisted of a linear gradient from 35 to 45% (v/v) solvent B in 18 min, followed by a linear gradient from 45% to 55% (v/v) solvent B in 5 min and isocratic elution at 55% solvent B for 7 min. GST subunits were detected with a 2140 Rapid Spectral Detector (Pharmacia, Uppsala, Sweden) operating at 214 nm.

Statistical analysis

Data are presented as means ± SD. Statistical differences were determined by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test and an orthogonal polynomial test [29]. A probability of $P \leq 0.05$ was considered significant.

Results

Clinical observations

Two rats in the group given the highest dose of *trans*-anethole (500 mg/kg) were found dead after 2 days. After dissection it was noticed that in both animals the stomach was stuffed with food while the intestines were empty. The third animal survived, but biotransformation enzyme activities were not determined. Therefore, this group was excluded from the experiment.

Treatment with eugenol or *trans*-anethole resulted in an apparent dose-related decrease in body weight in comparison with control rats, although the differences were not statistically significant (Table 1).

Relative liver weights of *trans*-anethole-treated rats increased with dose to 6 and 11% above those of the controls at the 125- and 250-mg/kg doses, respectively. The change in relative liver weight of the highest dose group was statistically significant when compared with the control group. For eugenol-treated rats, there were no significant changes in relative liver weight in comparison with the controls (Table 2).

Administration of *trans*-anethole or eugenol had no effect on the haematological parameters measured (numbers of erythrocytes, leucocytes and platelets, haemoglobin content and haematocrit). In eugenol- and *trans*-anethole-treated rats, plasma ALAT and ASAT activities were not different from those of the controls. All values fell within the range of control values (Table 3).

Table 1. Body weights of rats treated with *trans*-anethole or eugenol.

Treatment	Dose (mg/kg body weight)	Body weight (g) on day: 0	6	10
None (control)	0	246.0 ± 12.1	266.7 ± 16.3	283.0 ± 18.1
trans-Anethole	125	243.7 ± 8.5	260.7 ± 11.7	270.3 ± 16.2
	250	244.0 ± 3.6	255.3 ± 5.5	265.0 ± 9.6
Eugenol	250	244.7 ± 10.2	258.0 ± 21.9	275.0 ± 19.3
	500	242.0 ± 10.1	264.3 ± 5.9	270.7 ± 6.4
	1000	250.0 ± 22.3	249.3 ± 33.7	257.7 ± 40.4

Rats (three per treatment group) were treated with *trans*-anethole or eugenol for 10 consecutive days. Values are means ± SD.

Table 2. Relative liver weights and liver cytochrome P-450 content of rats treated with *trans*-anethole or eugenol.

Treatment	Dose (mg/kg body weight)	Relative liver weight (%)	Total P-450 (pmol/mg protein)
None (control)	0	3.9 ± 0.1	493 ± 128
trans-Anethole	125	4.2 ± 0.2	470 ± 57
	250	4.4 ± 0.2*	464 ± 28
Eugenol	250	4.1 ± 0.1	568 ± 23
	500	4.1 ± 0.0	523 ± 38
	1000	4.2 ± 0.4	585 ± 122

Rats (three per treatment group) were treated with *trans*-anethole or eugenol for 10 consecutive days. Livers were collected 24 h after the last dose. Values are means ± SD, and an asterisk indicates a significant difference from the control group (0 mg/kg) at $P \leq 0.05$.

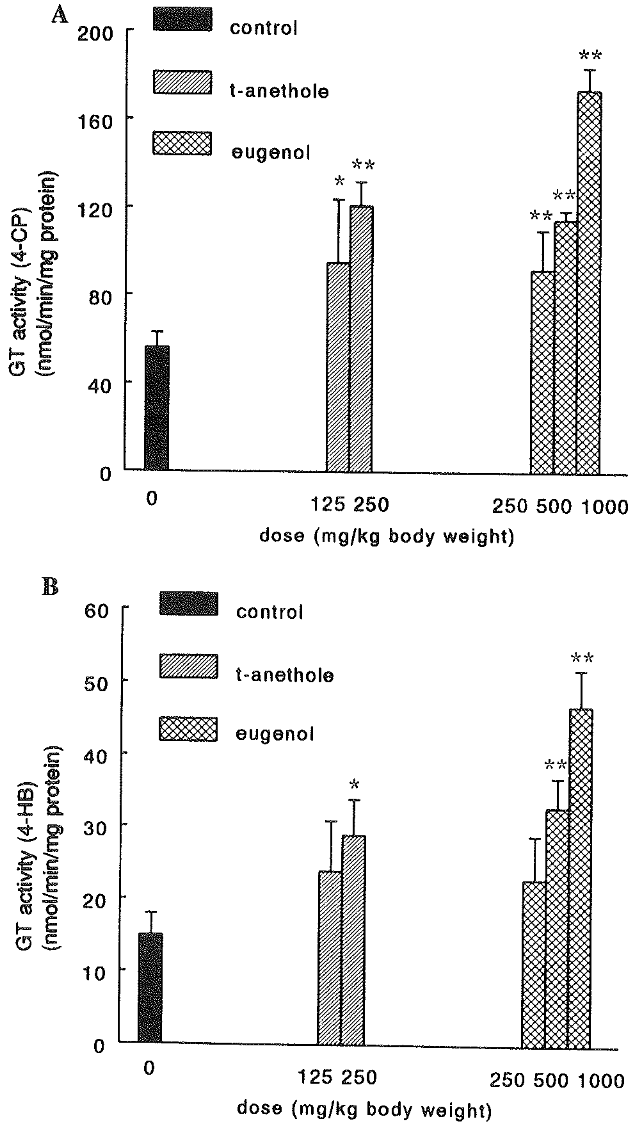

Fig. 3. Glucuronyl transferase (GT) activity towards the substrates (a) 4-chlorophenol (4-CP) and (b) 4-hydroxybiphenyl (4-HB) in liver microsomes of rats treated by gavage with *trans*-anethole (125 or 250 mg/kg) or eugenol (250, 500 or 1000 mg/kg) for 10 days. Values are means for three animals/group and range bars indicate the SD. Asterisks indicate significant differences from the control group at $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

Effects of trans-anethole and eugenol on phase-I enzyme activities

Total cytochrome P-450 contents of liver microsomes of rats treated with *trans*-anethole were not different from that of the controls. Treatment with eugenol resulted in slightly higher total cytochrome P-450 contents in comparison with controls (Table 2), but the increase was not statistically significant.

Administration of eugenol and *trans*-anethole caused a dose-related induction in the activities of liver cytochrome P-450-dependent EROD and PROD, which became significant, in comparison with the controls, at 1000 mg eugenol/kg (Fig. 2). For 1000 mg eugenol/kg the activities were 2.5 and 3.4 times those of control values for EROD and PROD, respectively.

Effects of trans-anethole and eugenol on phase-II enzyme activities

The activity of GT towards 4-hydroxybiphenyl as substrate was significantly increased in rats treated with 250 mg *trans*-anethole/kg (2.0- fold versus controls) or 500 or 1000 mg eugenol/kg (2.3- and 3.2-fold, respectively). The activities of GT towards 4-chloro-

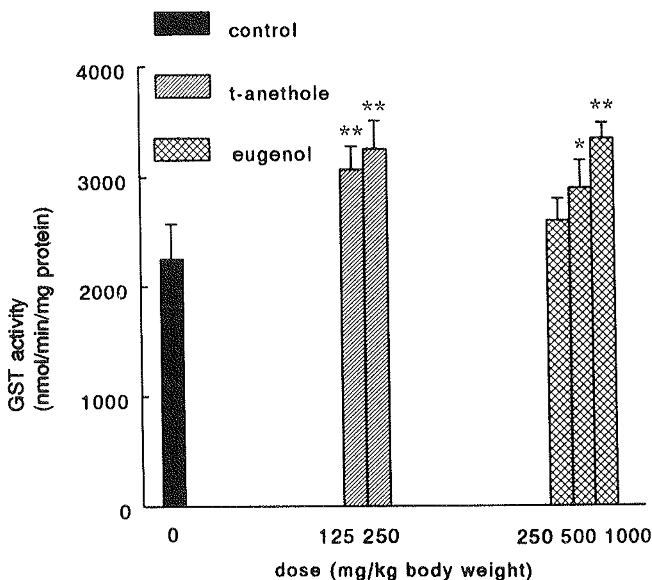

Fig. 4. GST activity in liver cytosol of rats treated by gavage with *trans*-anethole (125 or 250 mg/kg body weight) or eugenol (250, 500 or 1000 mg/kg) for 10 days. Values are means for three animals/group and range bars indicate the SD. Asterisks indicate significant differences from the control group at $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

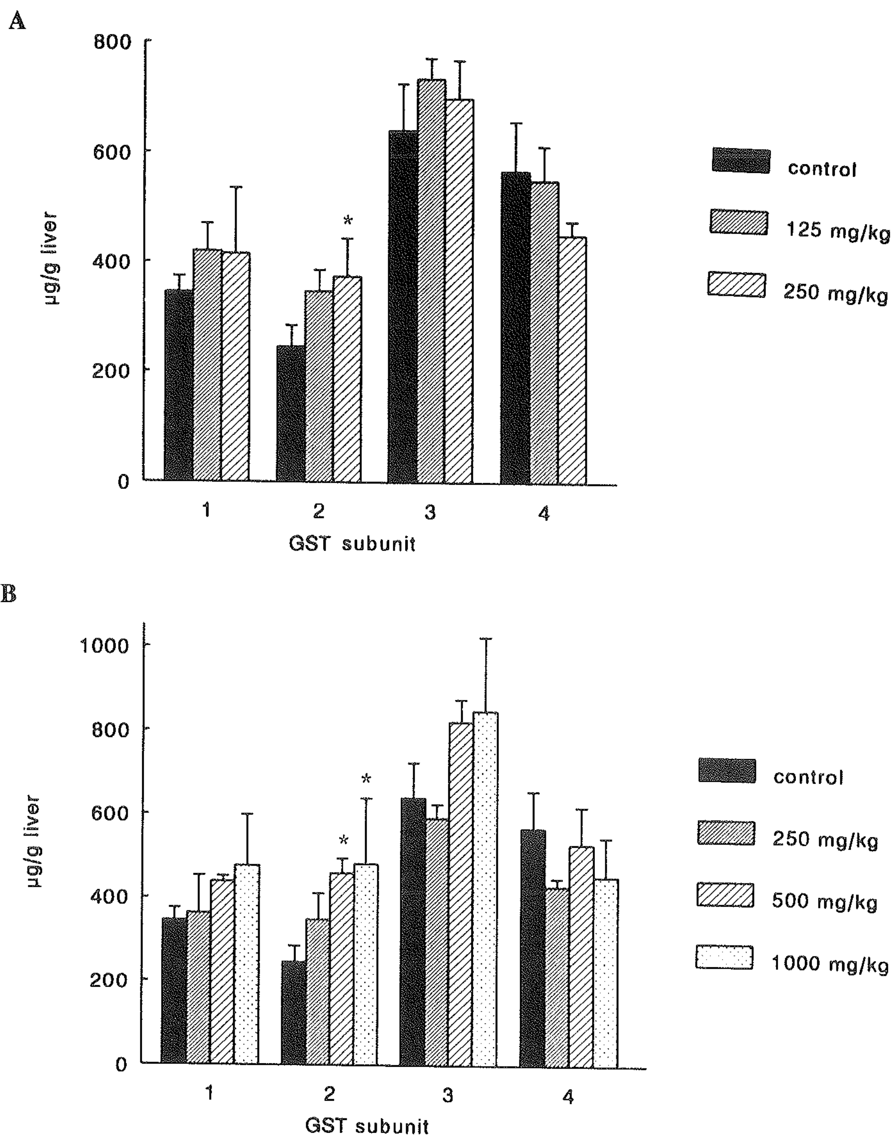

Fig. 5. Quantification of GST subunits in liver cytosol of rats treated by gavage for 10 days with (a) *trans*-anethole at 125 or 250 mg/kg body weight, or (b) eugenol at 250, 500 or 1000 mg/kg body weight. Values are means for three animals/group, and range bars indicate the SD. Asterisks indicate significant differences from the control group at $^{*}P \leq 0.05$.

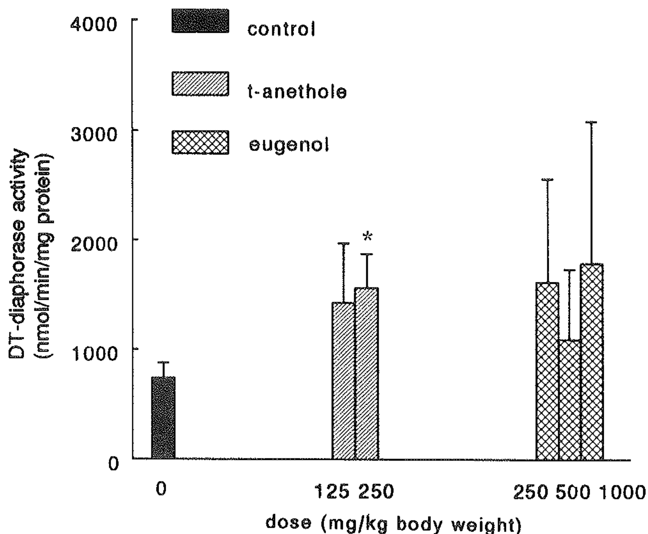

Fig. 6. DT-diaphorase (DTD) activity in the liver cytosol of rats treated by gavage with *trans*-anethole (125 or 250 mg/kg body weight) or eugenol (250, 500 or 1000 mg/kg) for 10 days. Values are means for three animals/group, and range bars indicate the SD. An asterisk indicates a significant difference from the control group at $^{*}P \leq 0.05$.

phenol as substrate were significantly increased in all rats treated with *trans*-anethole (1.7 - 2.1-fold) or eugenol (1.6 - 3.1-fold) (Fig. 3).

There was a significant enhancement of liver GST activity towards 1-chloro-2,4-dinitrobenzene by administration of 125 or 250 mg/kg *trans*-anethole (1.4- and 1.4-fold) in comparison with control rats. In the liver cytosol of rats treated with eugenol a dose-related enhancement of GST activity was observed. The enhancement became significant at 500 mg eugenol/kg when compared with the control group. Administration of 500 or 1000 mg eugenol/kg resulted in a 1.3-fold and 1.5-fold induction, respectively (Fig. 4). After separation of GST subunits significantly increased levels of GST subunit 2 were found in the liver of rats treated with eugenol (500 or 1000 mg/kg body weight) or *trans*-anethole (250 mg/kg body weight) (Fig. 5).

In eugenol-treated rats DTD activities were elevated 2.2-, 1.5- and 2.4-fold in the 250-, 500- and 1000-mg/kg dose groups, respectively, although none of these increases was statistically significant. In *trans*-anethole-treated rats, DTD activities were elevated

1.9- and 2.1-fold for the 125- and 250-mg/kg dose groups, respectively. The increase was statistically significant for the higher dose group when compared with the control group (Fig. 6).

Discussion

In experimental studies with laboratory animals, a wide variety of natural compounds has been shown to protect against the toxic and neoplastic effects of chemical carcinogens. Although a single mechanism cannot account for all forms of chemoprotection, the induction of electrophile processing phase-II enzymes (e.g. GST, GT and DTD) is a major protective mechanism. Molecular biological work has revealed some of the regulatory mechanisms underlying the induction of drug-metabolizing enzymes. Phase-II and other enzymes comprise a set, the co-ordinated expression of which is in part under the control of the aryl hydrocarbon hydroxylase [*Ah*] gene locus [30]. Rushmore and Pickett [31] postulated that planar aromatic compounds are bound by the *Ah* receptor and are translocated into the nucleus. In the nucleus the liganded receptor interacts with a xenobiotic regulatory element sequence, present in the CYP1A1 gene as well as in the GST subunit 1 gene, which results in a co-expression of both CYP1A1 and GST subunit 1. For the GST subunit 1 gene, Rushmore and Pickett [32] also describe an antioxidant-responsive element, which is activated by phenolic antioxidants, and results in induction of GST subunit 1, without, however concomitant induction of cytochrome CYP1A1.

Monitoring of phase-II enzyme induction has permitted the isolation and identification of new anticarcinogens [33]. In order to establish the chemopreventive capacities of eugenol and *trans*-anethole, we examined the effects of oral administration of these compounds on drug-metabolizing enzyme activities: both eugenol and *trans*-anethole induced a variety of drug-metabolizing enzymes in rat liver.

In the present study eugenol caused no significant increase in total microsomal cytochrome P-450, although cytochrome P-450-dependent EROD and PROD activities were slightly enhanced in comparison with the controls. The enhancement became statistically significant only at the highest dose (EROD and PROD activities elevated by 2.5- and 3.4-fold, respectively). EROD appears to be highly specific for cytochromes of the CYP1A family in a wide variety of species, whereas PROD appears to be mediated primarily by the CYP2B family [34,35,36,37]. In the present study the induction of CYP1A and CYP2B activity is quite small since inductions of P-450 enzyme activities can be more than 50-fold [38].

GT and GST activities were significantly and dose-relatedly enhanced following the administration of eugenol. This is in line with the results of Yokota *et al.* [39], who found an inducing effect of eugenol towards the activities of UDP-glucose dehydrogenase (1.6-fold), total GST (2.4-fold) and GT (2.7-fold) in rat liver after dietary administration of 3% (w/w) eugenol for 13 weeks. However, Yokota *et al.* [39] used a rather unusual dosing schedule: in the eugenol-treated group, diets containing 1, 3 and 5% (w/w) eugenol were changed several times during the experiment (week 1, 1% eugenol in the diet; week 2, 5% eugenol in the diet; week 3-8, 3% eugenol in the diet; week 9-10, 1% eugenol in the diet; week 11-12, 3% eugenol in the diet; week 13, 0% eugenol in the diet). In a second experiment diets containing 1, 3 or 5% (w/w) eugenol were fed to rats for 23 days. Dose-related enhancements of UDP-glucose dehydrogenase, total GST and GT activities towards various xenobiotics were observed [39]. Zheng *et al.* [40] also found a significant activity of eugenol as an inducer of total GST activity in the mouse liver (1.3-fold) and small intestine (2.4-fold) after oral administration of 20 mg eugenol per animal once every 2 days for a total of three administrations.

In order to investigate which GST isoenzymes have been induced, GST subunits have been quantified. Quantification of these subunits showed that GST induction was primarily caused by a dose-related and significant induction of GST subunit 2, although subunits 1 and 3 were also enhanced (but not significantly). The induction pattern of GST subunits by eugenol resembles the subunit induction pattern by benzyl and allyl isothiocyanate, with which a preferential enhancement of GST subunit 2 was also found [41,42].

In eugenol treated rats, DTD activities were elevated to approximately two-fold, although these increases were not statistically significant.

The above results indicate that administration of eugenol caused a considerable induction of the activity of phase-II enzymes since phase-II enzyme activities can be induced up to about three- to five-fold at most [43].

trans-Anethole, when administered for 10 days to rats, caused a small dose-related (although not significant) induction in liver EROD and PROD activities although no enhancement of total microsomal cytochrome P-450 was found. Reed and Caldwell [12] also found a small induction in EROD activity (69% increase over control) in hepatic microsomes of female Sprague-Dawley CD rats, treated ip with 300 mg/kg *trans*-anethole for 7 days. In contrast to the present results, they found a statistically significant increase in total microsomal cytochrome P-450 content (45% over the control). In a second study, *trans*-anethole was administered to male and female Sprague-Dawley CD rats at 0.25, 0.5 or 1.0% in the diet during 3 weeks. Cytochrome P-450 contents were elevated in female rats by 20, 42 and 69% over controls and in male rats by 5, 23 and 28% for the 0.25, 0.5 and 1.0% dose groups, respectively [12]. The discrepancy between the results of

Reed and Caldwell [12] and our results indicates that enhancement of cytochrome P-450 contents may be influenced by, for example, route of administration, strain and gender.

GT and total GST activities were significantly and dose-relatedly enhanced. Quantification of GST subunits showed a similar subunit induction pattern for *trans*-anethole as for eugenol: again a dose-related and significant induction of GST subunit 2 was seen.

DTD activity was found to be significantly enhanced in the liver cytosol of rats treated with 250 mg *trans*-anethole/kg (2.1-fold).

These results indicate that administration of *trans*-anethole has a more or less similar effect on drug-metabolizing enzymes as that of eugenol. *trans*-Anethole also caused a considerable induction of phase-II enzymes and a relatively small induction of CYP1A and CYP2B activity. In the literature no data on induction of phase-II enzymes by *trans*-anethole could be found.

Since a suitable candidate anticarcinogen should be anticarcinogenic at dose levels that are devoid of adverse effects, blood analyses were carried out to trace potential adverse effects. However, even at the highest dose levels no toxic effects were found: all clinical-chemical parameters did not significantly differ from those of the control group.

In *trans*-anethole-treated, but not in eugenol-treated rats, a dose-related increase in relative liver weight was observed. The increase in relative liver weight of the highest dose group (11% over control) was statistically significant when compared with the control group. These results are in agreement with the findings of Reed and Caldwell [12], who also found an increase in relative liver weight (8% over controls) in female rats treated ip with 300 mg *trans*-anethole/kg for 7 days. In another study, in which *trans*-anethole was administered to male and female rats in the diet for 3 weeks, relative liver weights increased with dose to 16% and 42% above control levels in female rats and to 13% and 27% above control levels in male rats for the 0.5% and 1.0% dose groups, respectively [12]. The observed increase in relative liver weight may be explained by an induction of microsomal enzymes, a phenomenon generally observed with non-genotoxic hepatocarcinogens [10].

From the results of the present study it appears that eugenol and *trans*-anethole are more effective inducers of phase-II enzymes than of cytochrome P-450 enzymes. This may be an indication that both eugenol and *trans*-anethole possess cancer preventive properties, which, in view of the apparent absence of toxic effects, may be relevant for humans. Indeed, for eugenol there are a few indications that it may have protective effects against chemical carcinogens. Eugenol was found to inhibit the mutagenicity of aflatoxin B1 and N-methyl N'-nitro-N-nitrosoguanidine in *Salmonella typhimurium* tester strain TA100 [44]. Mutagenicity of benzo[a]pyrene (B[*a*]P) in the Ames test using liver S-9 or microsomes prepared from rats that had been administered eugenol was decreased

in comparison with B[*a*]P-induced mutagenicity using untreated rat liver S9 or microsomes [45].

In contrast with the studies for eugenol, for *trans*-anethole no modulation studies of genotoxicity of known carcinogens could be found.

In the future much more work needs to be performed to establish more firmly the possible cancer preventive effects of eugenol and *trans*-anethole. Investigations in this area are currently being undertaken in our laboratory.

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CHAPTER 4

EFFECT OF EUGENOL ON THE GENOTOXICITY OF ESTABLISHED MUTAGENS IN THE LIVER

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Abstract

In order to verify whether eugenol has antigenotoxic potential *in vivo*, the influence of *in vivo* treatment with eugenol on the effect of established mutagens was studied. The effects of *in vivo* treatment of rats with eugenol was investigated in the *S. typhimurium* mutagenicity assay and in the unscheduled DNA synthesis assay (UDS assay) with established mutagens. In addition, in the single-cell gel electrophoresis (SCGE) assay, the effect of *in vitro* treatment with eugenol on the genotoxicity induced in Hep G2 cells by benzo(a)pyrene (B[a]P) was investigated. In the *S. typhimurium* mutagenicity assay, the mutagenicity of B[a]P was lower when liver S9 fractions were used that had been prepared from rats treated with eugenol p.o. (1000 mg/kg b.w.) than with liver S9 from control rats. For dimethylbenzanthracene (DMBA) no antimutagenic effect of liver S9 from eugenol-treated rats was found. In the UDS assay treatment of rats with eugenol p.o. did not cause a modification of UDS activity in hepatocytes isolated from these rats, after exposure of these cells *in vitro* to DMBA and aflatoxin B_1 (AFB1). In Hep G2 cells, four different treatment schemes of combinations of B[a]P and eugenol were examined: pre-treatment with eugenol, simultaneous treatment with eugenol and B[a]P, a combination of these (pre-treatment/simultaneous treatment), and post-treatment with eugenol. In the simultaneous treatment and in the pre- and simultaneous treatment an increase in the genotoxicity of B[a]P by eugenol was detected.

In the pre- and post-treatment no effect of eugenol on the genotoxicity of B[a]P was found. It can be concluded that the effect of eugenol on the genotoxicity induced by established mutagens is not univocal: *in vivo* treatment of rats with eugenol resulted in a reduction of the mutagenicity of B[a]P in the *S. typhimurium* mutagenicity assay, while in the UDS assay no effect of eugenol was found. *In vitro* treatment of cultured cells with eugenol resulted in an increase of genotoxicity of B[a]P. This means there is only limited support for an antigenotoxic potential of eugenol *in vivo*.

Introduction

Naturally occurring compounds can have important effects on the consequences of exposure to mutagens and carcinogens. Current scientific interest is directed towards the cancer-preventing potential of naturally occurring constituents of the diet [1]. As such, many foods (fruits and vegetables like cabbage, leeks, citrus, herbs and spices) and food ingredients (e.g. antioxidant vitamins, flavonoids, glucosinolates, organo-sulfur compounds) have been claimed to have antimutagenic or anticarcinogenic potential [2,3,4].

Alkenylbenzenes are an important group of naturally occurring food flavourings in herbs and spices. Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) is the main component of oil of cloves and is also present in the essential oils of many other plants, including cinnamon, basil and nutmeg.

Recently, in a subacute study we found a preferential induction of detoxifying phase-II biotransformation enzymes (glutathione S-transferase, GST, and glucuronyl transferase) by eugenol in rat liver *in vivo* [5]. Induction of these enzymes may be an indication that a compound (e.g. eugenol) possesses antimutagenic/anticarcinogenic properties [6].

In order to verify whether eugenol has antigenotoxic potential, the influence of *in vivo* and *in vitro* treatment with eugenol on the effect of established mutagens was studied. In the *S. typhimurium* mutagenicity assay the effect of liver S9 fractions isolated from eugenol-treated rats on the activation of two indirectly acting mutagens (benzo[a]pyrene, B[a]P, and 7,12-dimethyl-benz[a]anthracene, DMBA) was investigated. In the UDS assay hepatocytes were isolated from control and eugenol-treated rats and exposed *in vitro* to DMBA and aflatoxin B_1 (AFB1). The effect of *in vitro* treatment with eugenol on the genotoxicity of B[a]P was studied in the single-cell gel electrophoresis (SCGE) assay [7,8] with Hep G2 cells.

Materials and methods

Chemicals and reagents

All chemicals were of analytical reagent grade. Eugenol (purity 99%) was obtained from Janssen (Tilburg, The Netherlands). B[a]P was obtained from Sigma Chemical Company (St. Louis, MO, USA), DMBA from Eastman Kodak Co. (Rochester, NY) and AFB1 from Aldrich Chemie (Brussels, Belgium). [methyl-^{3}H]-Thymidine (46 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). Williams' Medium E was purchased from Flow Laboratories Ltd. (Herts, UK), Dulbecco's Modified Eagle Medium (DMEM) and gentamicin from Gibco BRL (Breda, The Netherlands) and foetal bovine serum from Integro B.V. (Zaandam, The Netherlands). Collagenase B was obtained from Boehringer Mannheim B.V. (Almere, The Netherlands). Seakem agarose (low-melting agarose) and SeaPlacque GTG agarose (normal-melting agarose) were obtained from FMC Bioproducts (Rockland, ME).

Animals and treatment

Male Wistar rats, b.w. approximately 160 g, were obtained from Charles River Wiga (Sulzfeld, Germany). Rats were housed in hanging type stainless-steel wire-mesh-bottomed cages, in an air-controlled room (22 ± 1°C) with 40-70% relative humidity and a light/dark cycle of 12 h. Rats were fed an open-formula basal diet for rodents that is commonly used at the TNO Nutrition and Food Research Institute. Food and water were available *ad libitum*.

Eugenol, dissolved in corn oil, was administered by gavage for 10 days at daily dose levels of 500 and 1000 mg/kg b.w. This dosing regimen for eugenol was similar to that used in a previous study [5]. The control group was given corn oil only. In all instances a volume of 10 ml/kg b.w. was administered.

Preparation of liver S9 fractions and S. typhimurium mutagenicity assay

The rats were killed with CO_2 at 24 h after the last dose. Livers were excised and homogenized in 3 volumes of 1.12 % (w/v) KCl in water. After centrifugation at 9000 x *g* for 12 min, part of the supernatant was pooled according to group, quickly frozen on dry ice and stored at -80°C (S9). The remaining supernatant was centrifuged at 100,000 x *g* for 90 min at 4°C. The supernatant representing the cytosolic fraction was separated and the pellet containing the microsomal fraction was resuspended in 1.12% KCl. Cytosolic and microsomal fractions were quickly frozen in liquid nitrogen and stored at -80°C until use. The mutagenesis assays were performed with *Salmonella typhimurium* strain TA100 according to Maron and Ames [9] with minor modifications. B[a]P and DMBA were tested by use of the liquid preincubation method. The mutagens were preincubated at 37°C with the bacteria and S9-fractions in the presence of a NADPH-generating system. After 30 min of

preincubation, 2 ml molten agar was added which contained 0.5 mM L-histidine and 0.5 mM biotin. The mixture was immediately poured onto minimal medium agar plates. The number of histidine revertant colonies was counted after 3 days of incubation at 37°C.

UDS assay

Hepatocytes were isolated from control and eugenol-treated rats (4 animals per group) by use of the two-step collagenase perfusion technique described by Seglen and Berry [10,11]. The viability of the cells was 72-93%, as determined by trypan-blue exclusion.

Aliquots of culture medium containing 4 x 10^5 viable hepatocytes were deposited onto 25-mm round Thermanox coverslips in 6-well tissue culture dishes. The culture medium consisted of Williams' medium E supplemented with 10 % foetal bovine serum, 2 mM L-glutamine and 50 μg/ml gentamicin. Cultures were incubated at 37°C under 5% CO_2 in air at 100% relative humidity. After 3 h the medium was replaced by 1.5 ml fresh medium containing also 5 x 10^{-5} M hydrocortison, 8 μg/ml insulin, 10 μCi/ml [methyl-^{3}H]-thymidine, and 0.1 μM AFB1 or 9.8 μM DMBA. The latter two compounds were added from stock solutions in DMSO. Control cultures were exposed to the vehicle (0.1% final volume) only. As negative control, hepatocytes of control animals (i.e. 0 mg/kg eugenol) were treated *in vitro* with DMSO. As positive control, hepatocytes of control animals were treated *in vitro* with DMBA or AFB1. Per eugenol treatment 4 animals were used. From each individual animal, hepatocytes were exposed in triplicate cultures to AFB1, DMBA or DMSO. After 18 h of incubation at 37°C, cells were washed with Williams' E medium, treated with a 1% sodium citrate solution for 10 min and fixed in ethanol-glacial acetic acid (3:1). Dried coverslips were mounted on glass slides. The slides were dipped in Kodak NTB-2 emulsion, exposed at -20°C for 7 days, developed in Kodak D19, fixed in Kodak fixative and rinsed with water. Slides were counterstained with haematoxylin and eosin, and embedded in DePeX. Slides were coded before analysis of [methyl-^{3}H]-thymidine incorporation in the hepatocytes. Fifty viable cells per slide were chosen at random and cytoplasmic labelling was determined by counting the grains in three different nucleus-size areas of cytoplasm adjacent to the nucleus. The mean cytoplasmic count was subtracted from the nuclear count to obtain the net number of nuclear grains.

Human hepatoma cell line (Hep G2), culture conditions and treatments

Hep G2 cells were kindly provided by Professor A.T. Natarajan and Dr. F. Darroudi, Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Leiden (The Netherlands). This cell line was originally established from human liver tumour biopsy [12]. Hep G2 cells were grown in 25-cm^2 cell culture flasks in DMEM, supplemented with 10% foetal bovine serum and 0.1% gentamicin. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. In the pre-treatment protocol, cells were treated with 3, 10, 30 or

100 μM eugenol for 15 h. After the cells were washed three times with PBS, 15 μM B[a]P was added for 24 h. In the pre-treatment/simultaneous treatment protocol, cells were first treated with 3, 10, 30 or 100 μM eugenol. After 15 h 15 μM B[a]P was added for 24 h. In the simultaneous treatment protocol, cells were treated with 3, 10, 30 or 100 μM eugenol and 15 μM B[a]P for 24 h. In the post-treatment protocol, cells were treated with 15 μM B[a]P for 24 h. After the cells were washed three times with PBS, 3, 10, 30 or 100 μM eugenol was added for 15 h. After treatment, cells were washed, trypsinized, resuspended in DMEM and spun down. The supernatant was discarded and cells placed on ice until use. Cells were checked for viability by trypan blue exclusion.

SCGE assay ('Comet'-assay)

The technique decribed by Pool-Zobel *et al.* [13] was followed with minor modifications. In short, cells were suspended in 0.7% LMA and distributed onto fully frosted microscope slides pretreated with 1% NMA and containing already one layer of 0.7% LMA. Subsequent lysis of the cells with EDTA was obtained in Triton X-100. Electrophoresis was carried out at 25 V, 300 mA for 20 min and the DNA was stained with ethidium bromide. Tail length (distance from the middle of the nucleus to the tail end) was measured automatically with the image analysis system Colourmorph (Perceptive Instruments, Orchard Lea, UK). Tail length was determined from 50 cells per slide; at least 2 slides per concentration were used. All experiments were repeated at least once.

In some experiments Hep G2 cells were frozen after treatment using a programmable freezer and stored in liquid nitrogen until analysis. Prior to conducting the comet-assay cells were thawed quickly. After thawing the viability was 70-80%.

Biotransformation enzyme activities

The activity of cytosolic GST was determined with 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* [14]. Cytosolic and microsomal protein-content was quantified by the method of Lowry *et al.* [15] with bovine serum albumin as a standard. Total microsomal cytochrome P-450 content was determined according to the method of Rutten *et al.* [16].

Metabolites of testosteron in microsomal preparations were determined and analysed by HPLC according to Wortelboer *et al.* [17] with minor modifications. In short, microsomes (1 mg protein) were incubated in 1 ml potassium-phosphate buffer (50 mM, pH 7.4) containing 3 mM $MgCl_2$, 1 mM NADPH and 250 μM testosterone at 37°C. After 10 min of incubation, the reaction was stopped with 6 ml dichloromethane and metabolites were extracted for analysis. 11ß-hydroxytestosterone (OHT) was used as an internal standard.

Bone marrow micronucleus assay

As positive control group, 4 rats were treated i.p. with 3 mg/kg mitomycin C once, 24 h prior to sacrifice. Femurs of eugenol-treated and control rats were dissected and bone marrow was flushed from the femoral cavity with foetal bovine serum. The collected cells were mixed with the serum and the cell suspension was centrifuged at 300 x *g* for 5 min. Cell pellets were resuspended and smears were air-dried, fixed in methanol, and stained with May-Grünwald Giemsa stain according to Schmid [18]. All glass slides were coded prior to analysis. For each animal, the number of micronucleated polychromatic erythrocytes (MPE) per 1000 polychromatic erythrocytes (PE) and the number of PE per 1000 erythrocytes were determined.

Statistical analysis

Data are presented as mean ± SD. Statistical differences were determined by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test and an orthogonal polynomial test [19]. For the micronucleus assay MPE-data were transformed prior to analysis by use of a square-root transformation to 'normalize' the counts [20]. If the result was significant ($P \leq 0.05$), asymptotic pairwise t-tests were performed (2-tailed).

Results

S. typhimurium mutagenicity assay

For B[a]P, incubation with liver S9 from rats treated with 1000 mg/kg b.w. eugenol markedly decreased the mutagenicity of B[a]P at all doses (20, 40 and 80 nmol/plate) as compared to liver S9 from control rats (Fig. 1).

For DMBA no antimutagenic effect of liver S9 of eugenol-treated rats was found (Fig. 2). At the highest concentration DMBA (120 nmol/plate) significantly higher values were found with liver S9 from eugenol-treated rats but this is probably due to an occasionally low value obtained with S9 from control rats, and is not considered biologically relevant.

UDS assay

The established mutagens DMBA and AFB1 gave a positive response in the UDS assay as expected (Fig. 3). In hepatocytes of control rats, DMBA and AFB1 gave a mean net nuclear grain count of 24.5 ± 8.1 (n=4), and 31.9 ± 1.8 (n=4), respectively. With both DMBA and AFB1, the mean nuclear grain count in hepatocytes of eugenol-pretreated rats did not differ significantly from that in hepatocytes of control rats. In hepatocytes of rats pretreated with 500 mg/kg eugenol, DMSO gave a significantly lower mean net nuclear grain count than in control hepatocytes ($P<0.05$).

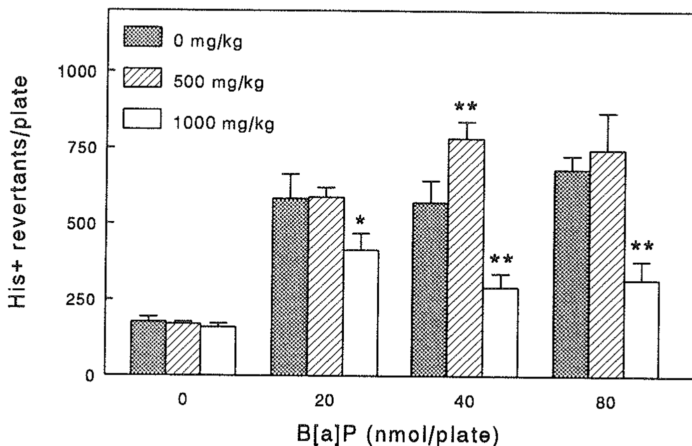

Fig. 1. Mutagenicity of B[a]P in the *S. typhimurium* mutagenicity assay with tester strain TA100 and liver S9 fractions prepared from rats treated orally with eugenol at 0 mg/kg b.w., 500 mg/kg b.w. and 1000 mg/kg b.w. for 10 days. Asterisks indicate significant differences from control rats (same concentration B[a]P) at $^{*}P<0.05$ and $^{**}P<0.01$. Mean ± SD.

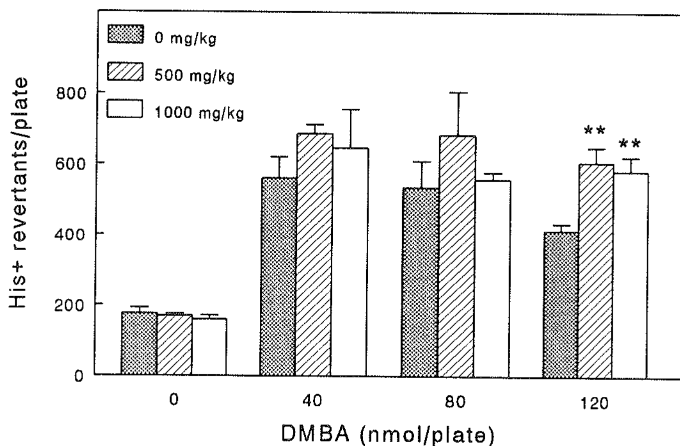

Fig. 2. Mutagenicity of DMBA in the *S. typhimurium* mutagenicity assay with tester strain TA 100 and liver S9 fractions prepared from rats treated orally with eugenol at 0 mg/kg b.w., 500 mg/kg b.w. and 1000 mg/kg b.w. for 10 days. Asterisks indicate significant differences from control rats (same concentration DMBA) at $^{**}P<0.01$. Mean ± SD.

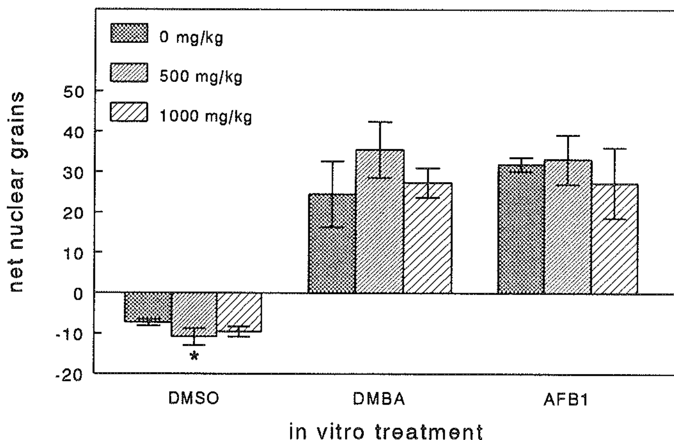

Fig. 3. Net nuclear grain count in hepatocytes of male Wistar rats treated orally with eugenol (0, 500 or 1000 mg/kg) for 10 days. Hepatocytes were treated *in vitro* with DMBA (9.8 μM) or AFB1 (0.1 μM). Data represent the mean $\pm$ SD for 4 animals. Asterisk indicates a significant difference from hepatocytes of control rats at $^{*}P \leq 0.05$.

Table 1. Tail length in Hep G2 cells treated with B[a]P or eugenol.

Eugenol (μM)	B[a]P (μM)	Exposure time (h)	Tail length (μm)
-	0 (DMSO)	24	19 $\pm$ 1
-	15	24	36 $\pm$ 1
0 (ethanol)	-	39	21 $\pm$ 2
3	-	39	19 $\pm$ 3
10	-	39	21 $\pm$ 1
30	-	39	20 $\pm$ 2
100	-	39	20 $\pm$ 2

Values are presented as mean values $\pm$ SD of two experiments (per experiment: 2 slides per concentration).

SCGE assay

Treatment of Hep G2 cells with the established mutagen B[a]P (15 μM) for 24 h gave a positive response in the SCGE assay (Table 1). Eugenol itself had no effect on the tail length (Table 1). In B[a]P-treated Hep G2 cells that were simultaneously or pre- and simultaneously treated with increasing eugenol concentrations, an increase in tail length was observed (Fig. 4). In contrast, pre- or post-treatment of Hep G2 cells with eugenol, does not

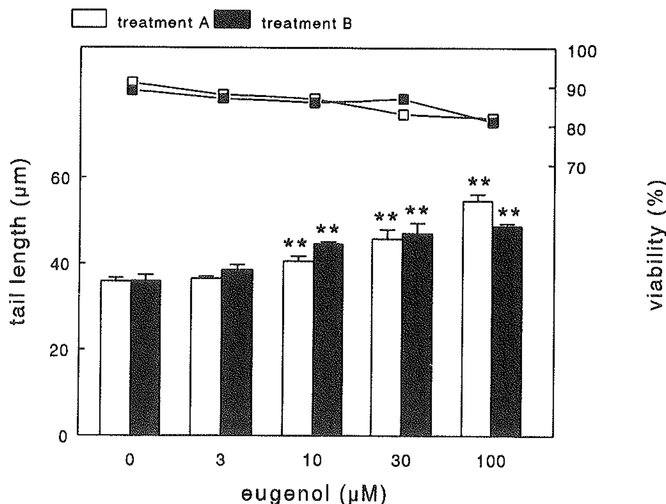

Fig. 4. Tail length and viability in Hep G2 cells treated with eugenol and B[a]P.
A. pre-treatment with eugenol for 15h, followed by similar treatment with eugenol and 15μM B[a]P for 24h; B. simultaneous treatment with eugenol and 15 μM B[a]P for 24h. Mean $\pm$ SD (4 slides per concentration). Asterisks indicate significant differences from the control (15 μM B[a]P, no eugenol) ($^{**}P < 0.01$).

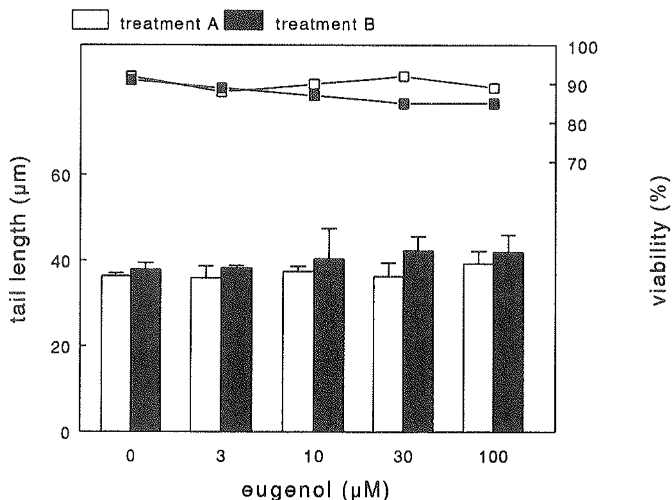

Fig. 5. Tail length and viability in Hep G2 cells treated with eugenol and B[a]P.
A. pre-treatment with eugenol for 15h, followed by 24h treatment with 15 μM B[a]P;
B. treatment with 15 μM B[a]P, post-treatment with eugenol for 15h. Mean $\pm$ SD (2 slides per concentration).

affect B[a]P-induced tail lengths (Fig. 5). Freezing of Hep G2 cells in liquid nitrogen after treatment and SCGE analysis later on, yielded the same results although the overall tail length was slightly higher (results not shown).

Biotransformation enzyme activities

There was a significant and dose-related enhancement of liver GST-activity towards 1-chloro-2,4-dinitrobenzene by administration of eugenol. Administration of 500 or 1000 mg eugenol/kg b.w. resulted in a 1.2-fold and 1.3-fold induction, respectively ($P<0.01$, Fig. 6). Total cytochrome P-450 content of liver microsomes of rats treated with eugenol was not significantly different from that of the controls (Table 2). Testosterone hydroxylation was significantly enhanced at the 16ß-site (indicating the activity of CYP2B1) in the liver of rats treated with 1000 mg/kg eugenol for 10 days (1.4-fold) as compared to their controls. No significant differences were detected in the hydroxylation reactions of testosterone at the 6ß, 7α, 16α and 2α site, which reflect activities of CYP3A, CYP1A1, CYP2B1 and CYP2C11, respectively (Table 2).

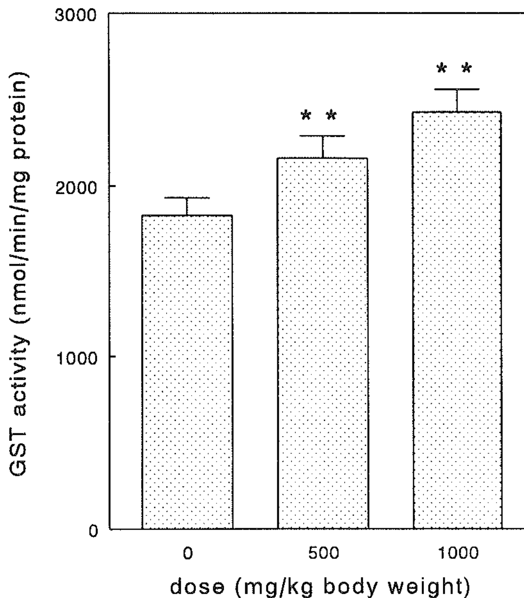

Fig. 6. Glutathione S-transferase (GST) activity in liver cytosol of rats treated orally with eugenol (0, 500 or 1000 mg/kg) for 10 days. Values are means for 4 animals/group and range bars indicate the SD. Asterisks indicate significant differences from the control group ($^{**}P<0.01$).

Table 2. Cytochrome P450 content and cytochrome P450-dependent hydroxylation of testosterone in microsomes prepared from livers of control rats and rats treated with eugenol for 10 days.

Eugenol (mg/kg)	P450 (pmol/ mg protein)	6ß-OHT (CYP3A)	7α-OHT (CYP1A1)	16α-OHT (CYP2B1)	16ß-OHT (CYP2B1)	2α-OHT (CYP2C11)
		(pmol/min/mg protein)				
0	675 ± 67	1054 ± 127	76 ± 6	1258 ± 387	75 ± 11	447 ± 124
500	688 ± 101	1184 ± 156	88 ± 10	1019 ± 181	85 ± 9	358 ± 50
1000	586 ± 78	1208 ± 188	88 ± 19	1061 ± 210	105 ± 21*	337 ± 86

P450 = total cytochrome P450 content; 6ß-OHT = the formation of 6ß-hydroxytestosterone (with corresponding cytochrome P450 isoenzyme activity); 7α-OHT = the formation of 7α-hydroxytestosterone; 16α-OHT = the formation of 16α-hydroxytestosterone; 16ß-OHT = the formation of 16ß-hydroxytestosterone; 2α-OHT = the formation of 2α-hydroxytestosterone. Values represent means ± SD (n=4). An asterisk indicates a significant difference from the corresponding control value (*$P < 0.05$).

Bone-marrow micronucleus test

Oral treatment with eugenol for 10 days had no effect on the incidence of MPE in the bone marrow cells (Table 3). The percentage of PE was slightly increased in rats given the highest dose level of eugenol (1000 mg/kg b.w.), although this increase was statistically not significant.

Table 3. Micronucleated polychromatic erythrocytes (MPE) in bone marrow cells of male Wistar rats treated orally with eugenol for 10 days. The positive control group was treated with mitomycin C once, 24 h before sacrifice.

Chemical	Dose (mg/kg b.w.)	MPE/1000PE[a]	Survival[b] (No. animal)	%PE[c]
Eugenol	0	1.25 ± 0.89	8/8	54.0 ± 8.3
	500	1.50 ± 0.93	8/8	54.5 ± 12.6
	1000	1.14 ± 1.07	7/8	60.2 ± 9.2
Mitomycin C	3	39.50 ± 3.70*	4/4	47.3 ± 8.2

[a] MPE per 1000 PE scored (mean ± SD).
[b] Number of animals surviving treatment over number of animals treated.
[c] Percentage of polychromatic erythrocytes of total erythrocytes (mean ± SD).
* $P<0.001$ versus control group.

Discussion

In a previous study, we found a preferential induction of biotransformation enzymes (GST, glucuronyl transferase over cytochrome P450 isoenzymes) by eugenol in rat liver *in vivo* [5]. In the present study, GST and cytochrome P450 activities were measured to confirm those results. Again an induction of GST activity (1.3-fold in rats treated daily with 1000 mg/kg for 10 days vs their controls) was found, although it was slightly smaller than that in the previous study (1.5-fold in rats treated daily with 1000 mg/kg for 10 days vs their controls). In both studies, total cytochrome P-450 contents of liver microsomes from rats treated with eugenol were not significantly different from those of the controls. In the present study the effects of eugenol on different isoenzymes of cytochrome P-450 was investigated further. For this purpose the hydroxylation of testosterone, which is catalysed by different isoenzymes of cytochrome P450 with a high degree of regio- and stereoselectivity, was investigated. Testosterone 7α-hydroxylation activity reflects the level of CYP1A1, 16α- and 16ß-hydroxylation activity the level of CYP2B1, 2α-hydroxylation activity the level of CYP2C11, while the formation of 6ß-hydroxytestosterone reflects the activity of CYP3A [17,21]. In microsomes of rats treated with 1000 mg/kg b.w. eugenol a significant increase in testosterone hydroxylation at the 16ß-site, but not at the 16α-site (both reflecting CYP2B1 activity) was found. This induction in CYP2B1 activity is in line with the results from the first study, where administration of 1000 mg/kg eugenol for 10 days caused a significant induction (3.4 times the control value) in the activity of liver cytochrome P-450-dependent 7-pentoxyresorufin dealkylation, which is considered to be mediated primarily by the CYP2B family. In both studies the induction of CYP2B activity is quite small in view of the fact that inductions of P-450 enzyme activities can in some cases be more than 50-fold [22]. No effects on other cytochrome P-450 isoenzyme activities were measured. From the results of both studies it can be concluded that eugenol is not an effective inducer of cytochrome P-450 enzyme activities. In both studies an induction of phase-II biotransformation enzymes was found. In general the fold-induction of phase-II biotransformation enzymes is low. For GST a small induction (in the present study 1.3-fold) may still be quite significant in view of of the relatively large amounts of GST present in most cells. The preferential induction of phase-II biotransformation enzymes (GST and glucuronyl transferase) may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [6].

For eugenol there are a few indications for protective effects against chemically induced mutagenesis. Eugenol inhibited the mutagenicity of aflatoxin B_1 and N-methyl-N'-nitro-N-nitrosoguanidine in *Salmonella typhimurium* tester strain TA100 [23]. Yokota *et al.* [24] found that use of S9 fractions or microsomes of rats treated 4 times with 800 mg/kg eugenol in 2 days resulted in suppressed mutagenicity of B[a]P the *S. typhimurium* mutagenicity

assay. In the same study they found a decrease to 81% and 29% of the control values for cytochrome P-450 content and arylhydrocarbon hydroxylase (AHH) activity in liver microsomes, respectively [24]. Yokota *et al.* [24] concluded that suppression of the mutagenicity of B[a]P is obviously caused by the reduction of AHH activity mediated by cytochrome P-450 in eugenol-treated rat liver microsomes. In the present study, incubation with liver S9 from rats treated with 1000 mg/kg b.w. eugenol markedly decreased the mutagenicity of B[a]P, which is in line with the results of Yokota *et al.* [24]. However, the explanation that this reduction is due to a suppression of AHH activity is not supported by the data from the present study because no significant change in CYP1A1 activity, which is specific for the metabolism of B[a]P (AHH activity) [25], was found. Another possible explanation for the reduction in mutagenicity of B[a]P by eugenol may be a decrease in epoxide hydrolase activity. This enzyme was not measured in the present study.

In order to study the possible antimutagenicity of eugenol via induction of GST induction, a modified *S. typhimurium* mutagenicity assay was used in which B[a]P or DMBA were preincubated with S9-mix containing glutathione [26]. No indications for an antigenotoxic potential of eugenol by GST induction were found [26].

No antimutagenic effect of liver S9 of eugenol-treated rats on the mutagenicity of DMBA was found. In the UDS assay, treatment of rats with eugenol did not cause a modification of DNA repair activity in hepatocytes exposed *in vitro* to DMBA and AFB1. Thus, except for B[a]P, there are no further indications for an antigenotoxic potential of eugenol *in vivo*.

In order to further study the effect of eugenol on B[a]P-induced genotoxicity, an *in vitro* experiment with different treatment schedules was performed with Hep G2 cells. The Hep G2 cell line is an immortalised liver cell line of human origin [12]. It has high B[a]P-metabolizing activity that converts B[a]P to intermediates that bind to DNA and are mutagenic [27]. In the SCGE assay with Hep G2 cells, pre-treatment with eugenol had no effect on the genotoxicity induced by B[a]P. In contrast, a significant enhancement in tail lenght of B[a]P-induced comets was found when high doses (10, 30, 100 μM) of eugenol were used in a simultaneous and a pre-treatment/simultaneous treatment protocol. As eugenol alone did not increase the comet length, it can be concluded that eugenol potentiates the genotoxicity of B[a]P.

Potentiation of the genotoxicity of B[a]P by eugenol in Hep G2 cells may be due to depletion of glutathione [28]. Babich *et al.* [28] found that intracellular glutathione levels were depleted to 87, 78 and 35% of control values in Hep G2 cells exposed for 24 h in growth medium containing 100, 200 and 250 μM eugenol, respectively. In the present study the highest eugenol concentration was 100 μM with an exposure time of 24 h (simultaneous

Table 4. Summary of the present studies on the modulation of genotoxicity by eugenol.

Test system	Mutagen/carcinogen Name	Concentration (treatment time)	Eugenol dose (treatment) *in vivo*	*in vitro*	Result[a]
UDS assay (rat hepatocytes)	DMBA	9.8 μM (18 h)	500 mg/kg (10x, po)[b]	n.a.	-
			1000 mg/kg (10x, po)	n.a.	-
	AFB1	0.1 μM (18 h)	500 mg/kg (10x, po)	n.a.	-
			1000 mg/kg (10x, po)	n.a.	-
S. typhimurium mutation assay (TA100)	B[a]P	20 nmol/plate	500 mg/kg (10x, po)[c]	(S9)	-
			1000 mg/kg (10x, po)	(S9)	↓
		40 nmol/plate	500 mg/kg (10x, po)	(S9)	↑
			1000 mg/kg (10x, po)	(S9)	↓
		80 nmol/plate	500 mg/kg (10x, po)	(S9)	-
			1000 mg/kg (10x, po)	(S9)	↓
S. typhimurium mutation assay (TA100)	DMBA	40 nmol/plate	500 mg/kg (10x, po)	(S9)	-
			1000 mg/kg (10x, po)	(S9)	-
		80 nmol/plate	500 mg/kg (10x, po)	(S9)	-
			1000 mg/kg (10x, po)	(S9)	-
		120 nmol/plate	500 mg/kg (10x, po)	(S9)	-
			1000 mg/kg (10x, po)	(S9)	-
SCGE assay (Hep G2 cell line)	B[a]P	15 μM (24 h)	n.a.	3 μM (pr, 15 h)	-
		15 μM (24 h)		10 μM (pr, 15 h)	-
		15 μM (24 h)		30 μM (pr, 15 h)	-
		15 μM (24 h)		100 μM (pr, 15 h)	-
	B[a]P	15 μM (24 h)	n.a.	3 μM (ps, 39 h)	-
		15 μM (24 h)		10 μM (ps, 39 h)	↑
		15 μM (24 h)		30 μM (ps, 39 h)	↑
		15 μM (24 h)		100 μM (ps, 39 h)	↑
	B[a]P	15 μM (24 h)	n.a.	3 μM (si, 24 h)	-
		15 μM (24 h)		10 μM (si, 24 h)	↑
		15 μM (24 h)		30 μM (si, 24 h)	↑
		15 μM (24 h)		100 μM (si, 24 h)	↑
	B[a]P	15 μM (24 h)	n.a.	3 μM (pt, 15 h)	-
		15 μM (24 h)		10 μM (pt, 15 h)	-
		15 μM (24 h)		30 μM (pt, 15 h)	-
		15 μM (24 h)		100 μM (pt, 15 h)	-

[a] ↓ = decrease of genotoxicity of an established mutagen/carcinogen by treatment with eugenol.
- = no effect of eugenol on the genotoxicity of an established mutagen/carcinogen.
↑ = increase of genotoxicity of an established mutagen/carcinogen by treatment with eugenol.
[b] hepatocytes of eugenol-treated Wistar rats (♂) have been used.
[c] S9 of eugenol-treated Wistar rats (♂) has been used.
n.a. = not applicable; AFB1 = aflatoxin B1; B[a]P = benzo[a]pyrene; DMBA = 7,12-dimethylbenz[a]anthracene; po = *per os*; pr = pre-treatment; ps = pre- and simultaneous treatment; pt = post-treatment; si = simultaneous treatment.

treatment) or 39 h (pre-treatment/simultaneous treatment). Based on the results of Babich *et al.* [28], a decrease of glutathione concentration with approximately 20% may be expected in the present study. Pre-treatment with eugenol did not result in an increased genotoxicity of B[a]P, which suggests that a possible decrease in glutathione concentration is rapidly restored after removal of eugenol.

Another possible explanation for the potentiation of the genotoxicity of B[a]P by eugenol in Hep G2 cells, is *in vitro* inhibition of GST activity by eugenol, because an inhibition of GST-activity implicates that B[a]P metabolites will not (or to a lesser extend) be detoxified by conjugation with glutathione. It is known that naturally occurring plant phenols (e.g. ellagic acid, ferrulic acid, caffeic acid, chlorogenic acid) are potent *in vitro* inhibitors of GST activity [29,30]. *In vitro* inhibition studies may be relevant to study the effect of eugenol on GST and glutathione.

In the present study a micronucleus test was included to exclude potential genotoxic effects of eugenol itself, since a compound that is to be used as an antimutagen should not be (geno)toxic by itself [31]. Indeed, no genotoxic damage in bone marrow cells was found in eugenol-treated rats (Table 3) which is in line with most but not all of the other reported bone marrow micronucleus tests with eugenol [32,33,34,35,36,37]. In the present micronucleus test, treatment with 1000 mg/kg b.w. eugenol resulted in an increase in the percentage of PE, although not significant, as compared to control rats. This increase was also found in our previous micronucleus study [37]. An increase of the percentage of PE may be caused by an increased production of erythrocytes, which could implicate that eugenol has hemolytic effects, although this has never been reported. Alternatively, eugenol may protect against bone marrow cytotoxicity induced by corn oil [38], which may also result in enhanced production of erythrocytes [39].

On the basis of the results obtained in the present studies (Table 4) it can be concluded that the results from the *S. typhimurium* mutagenicity assay indeed provide some support for an antigenotoxic potential of eugenol. However, no antigenotoxic effect of eugenol in the hepatocyte DNA repair assay and the SCGE assay was found. In the SCGE assay a potentiation of B[a]P-induced genotoxicity was found. Thus, there is no evidence for an univocal antimutagenic potential of eugenol *in vivo* and *in vitro*.

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CHAPTER 5

ANTIMUTAGENICITY OF EUGENOL IN THE RODENT BONE MARROW MICRONUCLEUS TEST

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Abstract

The antimutagenic effect of eugenol on the mutagenicity of cyclophosphamide (CP), mitomycin C (MMC), ethyl methanesulphonate (EMS) and benzo[a]pyrene (B[a]P) was assessed in the rodent bone marrow micronucleus test using male Swiss mice. Oral administration of eugenol (0.4% in the diet) for 15 days was found to decrease significantly the frequency of micronucleated polychromatic erythrocytes (MPEs) elevated by CP. No effect was found on the frequency of MPEs elevated by MMC, EMS and B[a]P. The results indeed provide some support for antimutagenic potency of eugenol *in vivo*.

Introduction

Naturally occurring compounds can have important effects on the consequences of exposure to mutagens and carcinogens. Current scientific interest is directed towards the cancer-preventing potential of naturally occurring constituents of the diet [1]. As such many foods (fruits and vegetables, like cabbage, leeks, citrus, herbs and spices) and food ingredients (e.g. antioxidant vitamins, flavonoids, glucosinolates, organo-sulfur compounds) have been claimed to have antimutagenic or anticarcinogenic potential [2,3].

Alkenylbenzenes are an important group of naturally occurring food flavourings of herbs and spices. Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) is the main component of oil of cloves and is also present in the essential oils of many other plants, including cinnamon, basil and nutmeg.

Recently, in a subacute study, we found a preferential induction of detoxifying phase-II biotransformation enzymes (glutathione S-transferase and glucuronyl transferase) by eugenol in rat liver *in vivo* [4], which may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [5]. Indeed, for eugenol there are indications for protective effects against chemically induced mutagenesis *in vitro*. Eugenol inhibited the mutagenicity of aflatoxin B_1 and N-methyl-N'-nitro-N-nitrosoguanidine in *Salmonella typhimurium* tester strain TA100 [6]. In addition, microsomes or S9 prepared from rats that received eugenol decreased the mutagenic activity of benzo[a]pyrene in the Ames test in comparison to the microsomes or S9 from untreated rats [7,8].

An established antigenotoxic response in *in vitro* test systems should be verified in *in vivo* test systems [9], taking into account the limitations of target cell concentrations and exposure of target cells: if antigenotoxic potential *in vivo* is not evident, then classification as an antigenotoxic substance is not appropriate [10]. Therefore, it is of interest to investigate whether eugenol reveals its antimutagenic activity in mammalian cells *in vivo*. In this study, the rodent bone marrow micronucleus test was applied to evaluate the role of eugenol in modulating the genetic damage induced by two indirect mutagens (cyclophospamide (CP) and benzo[a]pyrene (B[a]P)) and two direct mutagens (mitomycin C (MMC) and ethyl methanesulphonate (EMS)).

Materials and methods

Chemicals

Eugenol (purity 99%; CAS No. 97-53-0) was obtained from Janssen Chimica (Tilburg, The Netherlands); cyclophospamide (CP; CAS No. 6055-19-2), mitomycin C (MMC; CAS No. 50-07-7), ethyl methanesulphonate (EMS; CAS No. 62-50-0) and benzo[a]pyrene (B[a]P; CAS No. 50-32-8) were obtained from Sigma Chemical Company (St. Louis, USA).

Animals and diets

Male young adult Swiss mice (Charles River CD-1 strain), weighing approximately 32 g, 6 weeks old, were obtained from Charles River Wiga (Sulzfeld, Germany). They were housed individually in sterilized Makrolon cages with a grid cover of stainless steel

Table 1. Composition of the eugenol diet and the control diet (adapted from Rutten and de Groot [11]).

Ingredients	Content (%)	
	Eugenol diet	Control diet
Casein	20.0	20.0
dl-Methionine	0.3	0.3
Wheat starch	63.5	63.5
Cellulose	5.0	5.0
Choline bitartrate	0.2	0.2
Mineral mixture (based on AIN-76A)	3.5	3.5
Vitamin mixture (based on AIN-76A)	1.0	1.0
$CaHPO_4$	1.5	1.5
Corn oil	-	5.0
Eugenol premix[a]	5.0	-

[a]The eugenol premix contained 12% eugenol and 88% corn oil (w/w).

and with a bedding of sterilized softwood chips, in an air-controlled room (24 ± 1°C) with a relative humidity of 72 ± 16 % and a light/dark cycle of 12 h.

The experiment comprised two groups of 30 mice, which were fed a pelleted purified diet (control group) or a pelleted purified diet containing 0.6% eugenol (nominal concentration) for 15 days (Table 1). This dose has been chosen on the base of feed studies with eugenol reported by the National Toxicology Program [12], in which this dose level was the no-observed adverse effect level. In the eugenol diet, corn oil is replaced by an eugenol premix containing 12% eugenol and 88% corn oil (w/w). The diets were provided as pellets prepared by using a laboratory pelletizer. After pelletizing, samples were taken for analyses for concentrations of eugenol. The pellets were stored at -20°C until use. Twice a week pellets were replaced. Diets and tap water were available *ad libitum*.

Analysis of eugenol in diets

A 10-g sample of pellets was pulverised and extracted with 50 ml chloroform. The mixture was filtered and the extract was analysed using capillary gas chromatography (GC) with flame ionisation detection. Quantitation of eugenol in the samples (Fig. 1) was obtained by comparing the peak area ratio of eugenol and an internal standard (n-dodecane) with those of reference solutions in chloroform containing known amounts of eugenol. All samples were analysed using a Perkin Elmer 8500 GC under the following conditions: column: fused silica 50 m x 0.50 mm, 0.21 μm crosslinked methyl silicone;

column temperature: 180°C; carrier: Helium, 32 psi; injection temperature: 250°C; injection method: split 1:20; injection volume: 1.0 μl; detector temperature: 250°C.

Conduct of bone marrow micronucleus test

After 14 days, five animals per group were treated i.p. with one of the following test compounds: CP (25 mg/kg b.w.), MMC (1.5 mg/kg b.w.), EMS (300 mg/kg b.w.) and B[a]P (250 mg/kg b.w.). CP, MMC and EMS were dissolved in saline; B[a]P was suspended in olive oil. Control animals were given the solvents only (physiological saline or olive oil). The application volume was 10 ml/kg b.w. At 24 h after treatment with a test compound mice were killed by cervical dislocation. Both femurs were dissected, and bone marrow was flushed from the femoral cavity with foetal calf serum. The collected

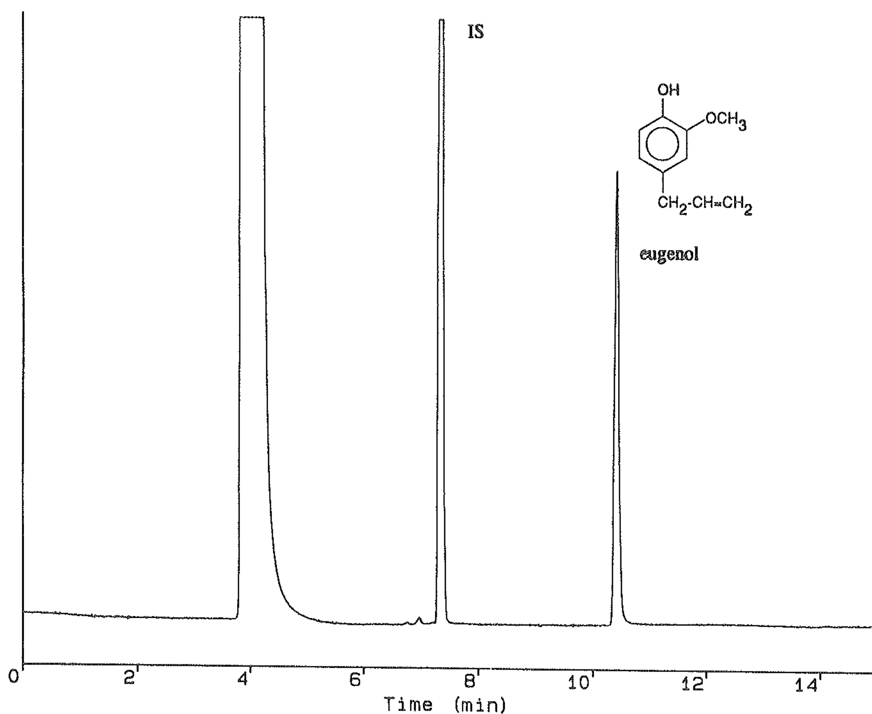

Fig. 1. Gaschromatogram of an extract of the eugenol diet (IS= internal standard, n-dodecane).

cells were mixed with the serum and the cell suspension was centrifuged at ca. 300 x *g* for 5 min. Cell pellets were resuspended and smears were air-dried, fixed in methanol, and stained with May-Grünwald Giemsa according to Schmid [13]. All glass slides were coded prior to observation. For each animal, the number of micronucleated polychromatic erythrocytes (MPE) per 1000 polychromatic erythrocytes (PE) and the number of PE per 1000 erythrocytes were determined.

Data were analyzed by two-way analysis of variance (ANOVA). Prior to analysis MPE-data were transformed using a square root transformation to 'normalize' the counts [14]. If the result was significant ($P<0.05$), asymptotic pairwise t-tests were performed (2-tailed).

Results

Chemical analyses showed that the eugenol diet (nominal concentration 0.6%) actually contained 0.4% eugenol and was stable at -20°C. The control diet contained no eugenol. At room temperature, the eugenol content of the diet decreased by 13% and 15% after 3 and 7 days, respectively.

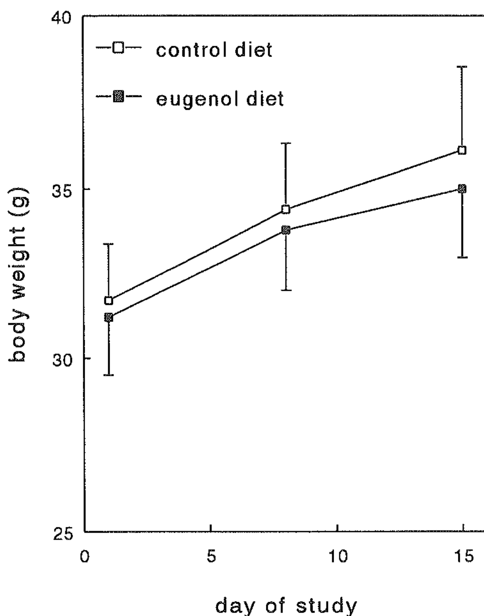

Fig. 2. Body weights of male Swiss mice fed a diet containing 0.4% eugenol or a control diet for 15 days (mean ± SD; n=30).

The protective effect of eugenol may be caused by a change in the activity of biotransformation enzymes involved, for instance induction of aldehyde dehydrogenase activity. Induction of this enzyme activity stimulates the formation of carboxyphosphamide from aldophosphamide [16]. This implies that less aldophosphamide will be converted into cytotoxic metabolites.

Another explanation for the protective effect of eugenol is the possible involvement of its antioxidant and scavenging properties. Eugenol has been found to be an inhibitor of lipid peroxidation and an antioxidative agent [17]. Scavenging of reactive molecules represents one of the approaches in antimutagenesis and anticarcinogenesis [18]. Therefore, eugenol protection against the clastogenic effects of CP could arise from the scavenging ability of eugenol to trap hydroxyl radicals originating from metabolites of cyclophosphamide with an OH functional group. A similar decrease in MPEs induced by CP has been described for other antioxidants like stobadine, also a scavenger of hydroxyl radicals [19].

For B[a]P, no significant decrease in MPEs was found in mice fed the eugenol diet. This is not consistent with the results from Yokota *et al.* [7] and Rompelberg *et al.* [8] who showed that microsomes or S9 prepared from rats that received eugenol significantly decrease the mutagenic activity of B[a]P in the Ames test in comparison to the microsomes or S9 from untreated rats. This discrepancy between the results of the microbial mutagenicity assay and the in vivo rodent mutagenicity test may be due to the underlying differences in the type of drug-metabolizing enzymes active in each system: in animal studies, phase-I and phase-II drug-metabolizing enzymes are operative while in the Ames test, only the enzymes which utilize NADPH or NADH, such as quinone reductase and monooxygenases are operative. The Ames test does not include cofactors for conjugating enzymes. Differences between the results of the present study and the results of the Ames test may further be explained by species differences between mouse and rat (rat microsomes were used in the Ames test). In an earlier study an induction of glutathione S-transferase- and glucuronyl transferase-activities was found in the liver of Wistar rats after oral treatment with eugenol [4]. However, induction of these enzyme activities (not measured in the present assay) apparently does not lead to detoxification of B[a]P via conjugation. Another possibility is that the decrease in MPEs is not statistically significant because of the sub-optimal sampling time. For B[a]P, a sampling time longer than 24 hours may be optimal [20], although Vanparys *et al.* [21] found that B[a]P showed a peak incidence of PEs at 24 h. In the present study only one sampling time for all four mutagens was used because an equal treatment with eugenol for all four mutagens was preferred.

No effect was found on the MPE frequency induced by the direct alkylating agents MMC and EMS in mice fed the eugenol diet.

In the present study, eugenol itself (680 mg/kg/day for 15 days) had no effects on the incidence of MPEs. This is consistent with most of the other bone marrow studies with eugenol [22,23,24]. In two studies, i.p. administration of eugenol resulted in induction of MPEs [25,26]. Oral administration caused only induction of MPE in very high doses (14,794.4 mg/kg) [25]. Thus, there is only limited evidence for the genotoxicity of eugenol in rodent bone marrow assays.

It is remarkable that consumption of the eugenol diet resulted in an increase in the percentage of PE in all treatment groups (significant only for mice treated with CP and EMS) as compared to mice fed the control diet. An increase of the percentage of PE may be caused by an increased production of erythrocytes. This could indicate that eugenol has hemolytical effects, although this has never been reported. Alternatively, eugenol may protect against possible bone marrow cytotoxicity induced by corn oil, which was present in both diets (Table 1). This may also result in enhanced production of erythrocytes [27].

To summarize, the present results indicate that eugenol indeed has some antimutagenic potential in vivo. In the future, more studies are needed to establish more firmly the possible antimutagenic effects of eugenol *in vivo*.

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DNA isolation and preparation of microsomes and cytosol

Livers were cut into pieces and homogenized in buffer (250 mM sucrose, 100 mM EDTA, pH 7.4); per gram liver 9 ml was used (all volumes are expressed per g liver as starting material). The homogenate was centrifuged (1000 x *g*, 10 min, 4°C). The supernatant was used for preparation of microsomes and cytosol and the pellet for DNA isolation. The supernatant was first centrifuged at 9000 x *g* for 30 min at 4°C and then at 100,000 x *g* for 90 min at 4°C. The supernatant containing the cytosolic fraction was separated and the pellet containing the microsomal fraction was resuspended in 1 ml 144 mM KCl (pH 7.4). Cytosolic and microsomal fractions were quickly frozen on solid CO_2 and stored at -80°C until analysis.

Liver DNA was isolated as described by Roggeband *et al.* [15]. Extractions of DNA were carried out with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and chloroform-isoamyl alcohol 24:1, v/v). The DNA was precipitated by addition of 0.1 volume of 3M sodium acetate, pH 6.0 and 3 volumes of ethanol at -20°C, washed with 70% ethanol at -20°C and dried *in vacuo*. The DNA was dissolved in 700 μl TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and used for the mutation assay. λDNA was rescued from the preparations by use of *in vitro* packaging extracts (Giga-Pack Gold II, Stratagene, La Jolla, CA, USA). For each sample, aliquots of DNA (5 μl) were mixed with half of the packaging extracts and incubated at 25°C for 6 h.

For determination of adducts, the DNA was dissolved in 3.0 ml TE buffer and treated with a mixture of 22.5 μl RNAse A (50 μg/ml) and 22.5 μl RNAse T1 (50 U/ml) for 90 min. Extractions of DNA were carried out with equal volumes of phenol, phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and chloroform-isoamyl alcohol (24:1, v/v). The DNA was precipitated, washed and dried as described above. The DNA was dissolved in 5 mM sodium acetate, pH 6.0.

Mutation assay

A culture of *E. coli C lacZ*⁻*rec*A⁻*gal*E⁻ harbouring a *gal*K⁻ and *gal*T⁻ expressing plasmid (pAA119) [16] was grown at 37°C to OD_{709}=0.6 in LB medium (Gibco BRL, Paisley, Scotland) supplemented with 10 mM $MgSO_4$, 0.2% maltose and 60 μg/ml ampicillin. Packaged DNA was added to 700 μl of the bacterial suspension and incubated for 16 min at 30°C. After incubation 10 μl of the phage/bacteria mix was used for titration. The remaining phage/bacteria mix was suspended in 5 ml top-agar containing 2% LB, 0.8% agar, 10 mM $MgSO_4$, 60 μg/ml ampicillin, 0.3% fructose, and 0.3% phenyl-ß-D-galactopyranoside (P-gal). The mixture was poured onto 10-ml agar plates (containing 2% LB, 1.6% agar, 10 mM $MgSO_4$ and 0.3% fructose) and incubated overnight at 37°C. The resulting (mutant) plaques were picked out and tested on X-gal containing plates to confirm their mutant status (Mientjes *et al.*, in preparation).

^{32}P-postlabelling analysis for the detection of B[a]P-DNA adducts

The ^{32}P-postlabelling assay was performed according to the nuclease P1 enhancement procedure [17]. Ten μg DNA was digested with 0.6 U micrococcal nuclease and 0.012 U spleen phosphodiesterase in a total volume of 12 μl 20 mM sodium acetate, 10 mM $CaCl_2$, pH 6.0 at 37°C for 3 h. After digestion 6 μl was used for quantification of the amount of input DNA by FPLC analysis [18]. The other 6 μl was dephosphorylated with 3 U nuclease P1 in the presence of 0.03 mM $ZnCl_2$ and 0.04 M sodium acetate, pH 5.0, at 37°C for 40 min. The reaction was terminated by the addition of 1.2 μl 0.5 M Tris. The remaining nucleotides were ^{32}P-postlabelled in a total volume of 13.2 μl in the presence of 2.85 MBq carrier-free [γ-^{32}P]ATP, 2.5 U T4 polynucleotide kinase, 100 mM Bicine, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine, pH 9.0, at 37°C for 40 min. Ten μl of the labelled digest was applied to a 20x10-cm PEI-cellulose sheet (Baker, Phillipsburg, NJ, USA) which was developed according to conditions suitable for B[a]P-DNA adducts in the following solvents: 1 M sodium phosphate, pH 6.0 (D1); 3 M lithium formate, 8.5 M urea, pH 3.5 (D3); 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0 (D4); 1.7 M sodium phosphate, pH 6.0 (D5). Between (two) developments the chromatograms were washed with water and air-dried. All chromatographic steps were carried out in subdued light to avoid photochemical decomposition of DNA adducts [18]. The ^{32}P-labelled adducts were visualized by autoradiography at -70°C for 15h with Kodak XAR-5 film and intensifying screens.

^{32}P-postlabelling analysis for the detection of eugenol-induced DNA adducts

The conditions for DNA digestion, nuclease P1 treatment and ^{32}P-labelling for analysis of eugenol-induced DNA adducts were the same as described above for B[a]P-DNA adducts. The solvents for chromatography were adapted to the conditions suitable for alkenylbenzene-DNA adducts [19,20,21]: 2.3 M sodium phosphate pH 5.7 (D1); 2.8 M lithium formate, 4.88 M urea pH 3.35 (D3); 0.34 M sodium phosphate, 0.21 M Tris-HCl, 3.75 M urea, pH 8.0 (D4); 1.7 M sodium phosphate, pH 6.0 (D5).

Quantitative analysis of the adduct levels

The relative amount of radioactivity in the adduct spots was determined by use of a Phosphor Imager system from Molecular Dynamics (Sunnyvale, CA, USA). The amount of background radioactivity was determined in a comparable blank area of the chromatogram close to the spots. The adduct levels were determined by comparison with standard samples analyzed concurrently with the chromatographic conditions for B[a]P-DNA adducts. The standards used for quantification were B[a]P diol-epoxide (BPDE) modified DNA samples in which the amount of B[a]P-DNA adducts had been determined by synchronous fluorescence spectrophotometry [18].

Biotransformation enzyme activities

Cytosolic and microsomal protein content was quantified by the method of Lowry *et al.*, with bovine serum albumin as a standard [22]. The activity of cytosolic glutathione S-transferase (GST) was determined with 1-chloro-2,4-dinitrobenzene as a substrate as described by Habig *et al.* [23]. Microsomal glucuronyl transferase (GT) activity was determined with 4-chlorophenol and 4-hydroxybiphenyl as substrates as described by Mulder and van Doorn [24].

Statistical analysis

Data are presented as means ± SD. Statistical differences were determined by two-way analysis of variance (ANOVA). Before analysis, data were transformed by log transformation to stabilize the variance. When the result was significant ($P \leq 0.05$), asymptotic pairwise t-tests with Bonferroni α-correction were performed (2-tailed) [25].

Results

Diet and body weight

Chemical analyses showed that the eugenol diet (nominal concentration 0.6%) contained actually 0.4% eugenol and was stable at -20°C [14]. The control diet contained no eugenol. At room temperature, the eugenol content of the diet had decreased by 13% and 15% after 3 and 7 days, respectively.

There was no statistically significant difference in food intake of mice on the eugenol diet (mean ± SD: 4.21 ± 0.40 g/mouse/day) versus mice on the control diet (mean ± SD: 4.26 ± 0.51 g/mouse/day). The eugenol diet resulted in an apparent growth retardation in comparison with the control group, although the differences were not statistically significant (Fig. 1).

Mutation assay

The established mutagen B[a]P significantly increased the *lac*Z mutant frequency in liver of mice fed the control diet (185.2 ± 66.7; mean ± SD) as compared to the vehicle control olive oil (71.3 ± 25.3); $P \leq 0.05$. Eugenol itself had no effect on the *lac*Z mutant frequency. No effect of eugenol was found on the *lac*Z mutant frequency induced by B[a]P (Table 2).

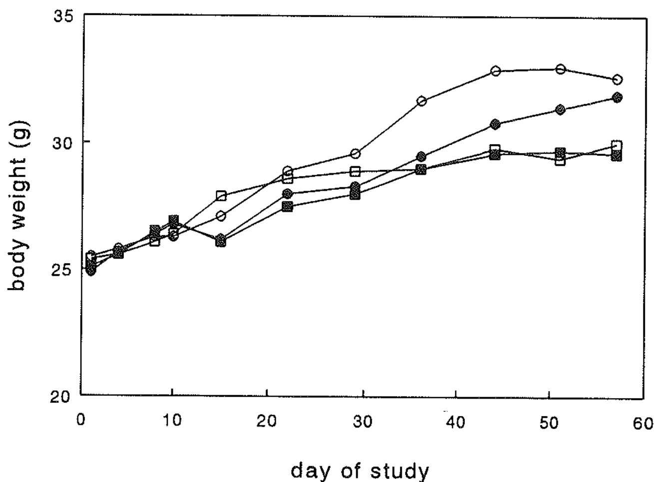

Fig. 1. Body weights of λ-*lac*Z-transgenic mice fed a diet containing 0.4% eugenol (□ , ■) or a control diet (○ , ●) for 58 days and treated i.p. with 100 mg/kg B[a]P (■ , ●) on day 10. Values are means of 4 animals.

^{32}P-postlabelling analysis with chromatographic conditions for B[a]P-DNA adducts

The adduct pattern for liver DNA of mice fed the control diet and treated with B[a]P was obtained by ^{32}P-postlabelling under chromatographic conditions suitable for B[a]P-DNA adducts, and is shown in Figure 2a. Four different spots were observed that were not detected on chromatograms obtained with samples of untreated mice (Fig. 2b). The quantitative results are presented in Table 3. The total adduct level in liver DNA of B[a]P-treated mice estimated by comparison with the B[a]P-standard samples, was 18.2 $\pm$ 8.2 adducts per 10^8 nucleotides. Adduct 1 co-chromatographed with the B[a]P-N^2-dG adduct of BPDE-treated DNA and represented 36% of the total amount of adducts. The identity of adducts 2, 3, and 4, representing respectively 10, 48 and 6% of the total, is not known. Dietary treatment with eugenol had no quantitative or qualitative effect on the formation of B[a]P-DNA adducts (Fig. 2c and 2d); the total adduct level was 17.1 $\pm$ 4.4 adducts/10^8 nucleotides and the adducts 1, 2, 3 and 4 represented 43, 10, 41 and 6% of the total amount.

^{32}P-postlabelling analysis with chromatographic conditions for alkenylbenzene-DNA adducts

Adduct patterns obtained by ^{32}P-postlabelling and chromatography under conditions suited for alkenylbenzene-DNA adducts, are shown in Figure 3. The adduct levels are presented in Table 4. In liver DNA from mice fed the eugenol diet without B[a]P, one

Table 2. *LacZ* mutant frequencies in liver of λ-*lacZ*-transgenic mice fed a diet containing 0.4% eugenol or a control diet for 58 days and treated i.p. with B[a]P on day 10.

B[a]P (mg/kg bw)	eugenol (% diet)	No. of phages analyzed	No. of mutants	MF[1] (x 10^6)	group mean[2] MF ± SD
0	0.0	521550	24	46.0	71.3 ± 25.3
		226062	24	106.2	
		330030	23	69.7	
		458280	29	63.3	
0	0.4	266760	19	71.2	63.9 ± 22.1
		347130	12	34.6	
		320625	20	62.4	
		389025	34	87.4	
100	0.0	301815	36	119.3	185.2 ± 66.7*
		195453	29	148.4	
		184509	50	271.0	
		415530	84	202.2	
100	0.4	309510	47	151.9	206.5 ± 48.6**
		205884	54	262.3	
		460161	105	228.1	
		239400	44	183.8	

Asterisks indicate a significant difference versus the corresponding control group (same diet, no B[a]P treatment) at *P ≤0.05 and **P ≤0.01.
[1] Mutant frequency (MF) x 10^6
[2] n=4

specific adduct spot was detected, which was absent in DNA from mice receiving the control diet (cf Fig. 3d, b). The level of this adduct, estimated by comparison with the B[a]P-standard samples, was 15.6 ± 6.4 adducts/10^8 nucleotides (Table IV, adduct 5). In liver DNA of mice fed the eugenol diet and treated with B[a]P this adduct was also observed in about the same quantity, 19.0 ± 4.5 adducts/10^8 nucleotides. Details of the formation of eugenol-DNA adducts will be published elsewhere (Steenwinkel *et al.*, in preparation). In these samples also four B[a]P-induced DNA adducts (6, 7, 8, and 9) were observed. The total amount of these adducts was 21.7 ± 6.3 adducts/10^8 nucleotides. In liver DNA samples of mice treated with B[a]P and fed the control diet, also these four adducts were observed. The adduct level of the four individual B[a]P adducts was higher (although not significantly) than in mice treated with B[a]P and fed the eugenol diet, resulting in a higher total amount of B[a]P-DNA adducts (although not significantly): 33.4 ± 13.0 adducts/10^8 nucleotides. When BPDE-modified standard DNA was analyzed, adducts 6, 7, and 9 were also observed, with adduct 6 being the main adduct (80%) (not shown).

Phase-II biotransformation enzyme activities

Dietary treatment with eugenol caused a significant, two-fold reduction of the liver GST activity towards 1-chloro-2,4-dinitrobenzene in comparison with control mice (Fig. 4). There was no significant effect of dietary treatment with eugenol on the activity of GT towards the substrates 4-chlorophenol or 4-hydroxybiphenyl (Fig. 5). Treatment with B[a]P (100 mg/kg b.w., i.p.) caused no significant change in liver GST activity (Fig. 4) and GT activity (Fig. 5) in comparison with control mice.

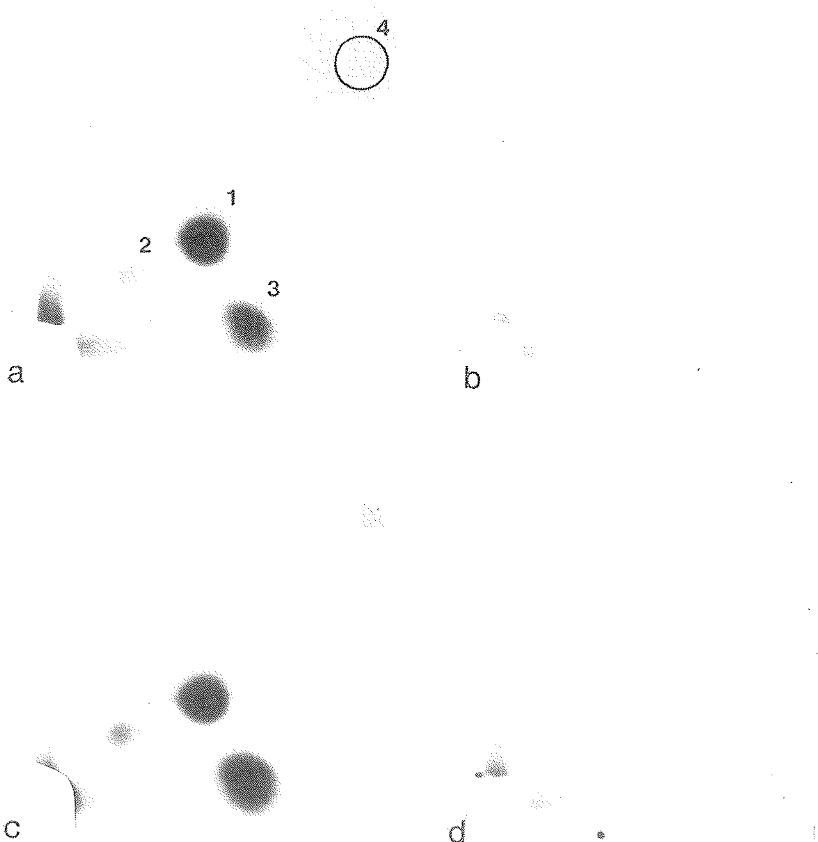

Fig. 2. Autoradiograms of ^{32}P-postlabelled DNA isolated from liver from λ-*lac*Z-transgenic mice (a) fed a control diet for 58 days and treated i.p. with 100 mg/kg B[a]P on day 10, (b) fed a control diet for 58 days, (c) fed a diet containing 0.4% eugenol and treated i.p. with 100 mg/kg B[a]P on day 10, and (d) fed a diet containing 0.4% eugenol; determined by ^{32}P-postlabelling analysis with chromatographic conditions for B[a]P-DNA adducts.

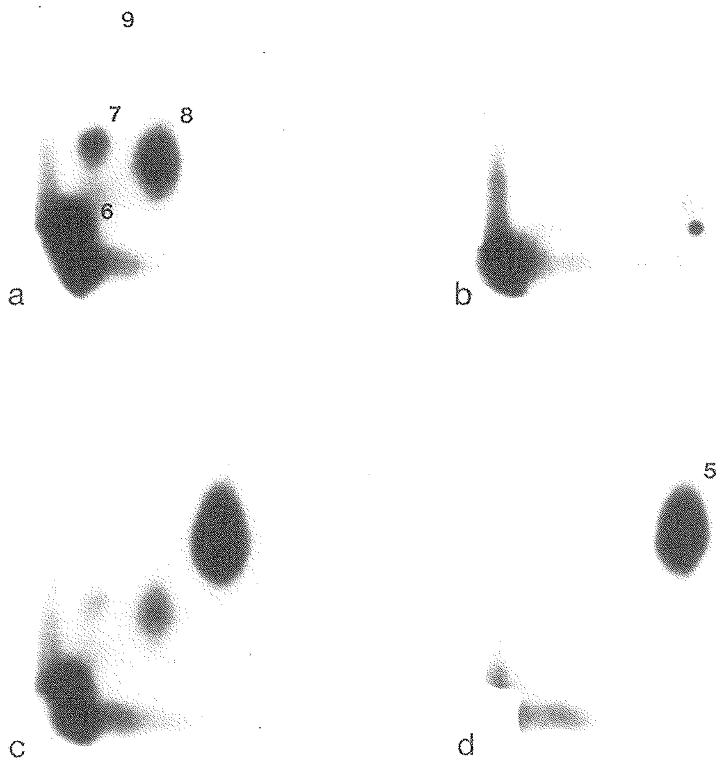

Fig. 3. Autoradiograms of ^{32}P-postlabelled DNA isolated from liver from λ-*lac*Z-transgenic mice (a) fed a control diet for 58 days and treated i.p. with 100 mg/kg B[a]P on day 10, (b) fed a control diet for 58 days, (c) fed a diet containing 0.4 % eugenol and treated i.p. with 100 mg/kg B[a]P on day 10, and (d) fed a diet containing 0.4% eugenol; determined by ^{32}P-postlabelling analysis with chromatographic conditions for alkenylbenzene-DNA adducts.

Table 3. DNA adduct levels (in adducts per 10^8 nucleotides) in liver DNA of λ-*lacZ*-transgenic mice fed a diet containing 0.4% eugenol or a control diet for 58 days and treated i.p. with B[a]P on day 10, determined by ^{32}P-postlabelling analysis with chromatographic conditions for B[a]P-DNA adducts.

B[a]P (mg/kg bw)	eugenol (% diet)	adduct 1	adduct 2	adduct 3	adduct 4	Total adduct level
0	0	0.5 ± 0.3 (38%)	0.4 ± 0.2 (31%)	0.1 ± 0.2 (9%)	0.3 ± 0.2 (22%)	1.3 ± 0.8 (100%)
0	0.4	0.4 ± 0.2 (41%)	0.3 ± 0.2 (32%)	0.1 ± 0.1 (12%)	0.2 ± 0.1 (15%)	1.0 ± 0.5 (100%)
100	0	6.6 ± 2.9 (36%)	1.9 ± 1.0 (10%)	8.7 ± 4.6 (48%)	1.1 ± 0.8 (6%)	18.2 ± 8.2 (100%)
100	0.4	7.4 ± 2.6 (43%)	1.7 ± 0.7 (10%)	6.9 ± 1.3 (41%)	1.1 ± 0.7 (6%)	17.1 ± 4.4 (100%)

Table 4. DNA adduct levels (in adducts per 10^8 nucleotides) in liver DNA of λ-*lac*Z-transgenic mice fed a diet containing 0.4% eugenol or a control diet for 58 days and treated i.p. with B[a]P on day 10, determined by ^{32}P-postlabelling analysis with chromatographic conditions for alkenylbenzene-DNA adducts.

B[a]P (mg/kg bw)	eugenol (% diet)	adduct 5	adduct 6	adduct 7	adduct 8	adduct 9	Total adduct level for adducts 6 - 9
0	0	1.6 ± 1.4	2.6 ± 2.8 (44%)	1.2 ± 1.0 (20%)	1.9 ± 2.1 (32%)	0.2 ± 0.1 (4%)	6.0 ± 5.9 (100%)
0	0.4	15.6 ± 6.4	1.9 ± 0.6 (42%)	1.0 ± 0.2 (22%)	1.4 ± 0.7 (31%)	0.2 ± 0.1 (5%)	4.4 ± 1.3 (100%)
100	0	1.5 ± 0.6	15.9 ± 7.7 (47%)	5.0 ± 1.8 (15%)	11.4 ± 3.9 (34%)	1.2 ± 0.6 (4%)	33.4 ± 13.0 (100%)
100	0.4	19.0 ± 4.5	10.8 ± 3.4 (50%)	3.3 ± 1.3 (15%)	6.5 ± 1.5 (30%)	1.0 ± 0.9 (5%)	21.7 ± 6.3 (100%)

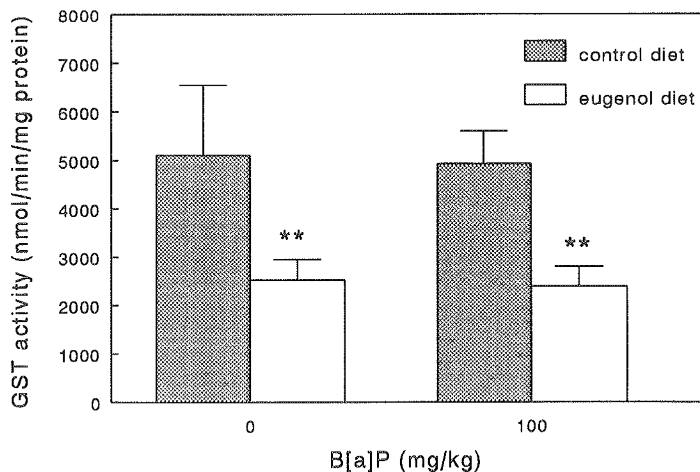

Fig. 4. Glutathione S-transferase (GST) activity in liver cytosol of λ-*lac*Z-transgenic mice fed a diet containing 0.4% eugenol or a control diet for 58 days and treated i.p. with B[a]P on day 10. Values are means ± SD. Asterisks indicate significant differences from the control group at $P \leq 0.01$.

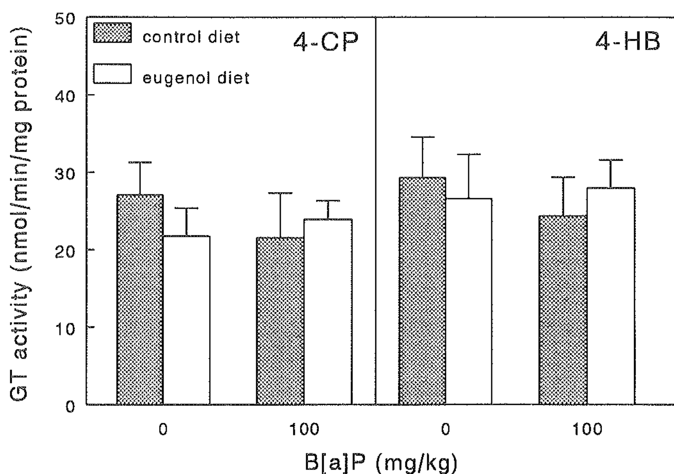

Fig. 5. Glucuronyl transferase (GT) activity towards the substrates 4-chlorophenol (4-CP) and 4-hydroxybiphenyl (4-HB) in liver microsomes of λ-*lac*Z-transgenic mice fed a diet containing 0.4% eugenol or a control diet for 58 days and treated i.p. with B[a]P on day 10. Values are means ± SD.

Discussion

In previous studies indications were found for an antigenotoxic potential of eugenol *in vitro* and *in vivo*. Yokota *et al.* [9] and Rompelberg *et al.* [10] showed that microsomes or S9 prepared from livers of rats that received eugenol significantly decrease the mutagenic activity of B[a]P in the Ames test in comparison to microsomes or S9 from untreated rats. It was also shown that eugenol is an inducer of phase-II drug metabolizing enzymes (GST and GT) *in vivo* [6,26,27]. The present study has been performed to further investigate whether eugenol reveals an antigenotoxic potential *in vivo*. When such an effect cannot be confirmed *in vivo* classification as an antigenotoxic substance is not appropriate [12].

Mutation assay

In the present study B[a]P was shown to be an *in vivo* genotoxin that is detectably mutagenic to the *lac*Z transgene: a clearly positive mutagenic response was observed for B[a]P in the λ-*lac*Z-transgenic mouse mutation assay 48 days after an i.p. injection of B[a]P (100 mg/kg b.w.). Dietary treatment with eugenol did not result in a significant decrease in mutant frequency induced by B[a]P. This is in line with the results of an *in vivo* micronucleus test performed by Rompelberg *et al.* [14]. In that study no effect was found of dietary treatment with eugenol on B[a]P-induced micronuclei in bone marrow of mice. The discrepancy between the *in vitro* and *in vivo* results may be due to the underlying differences in the type of metabolizing enzymes active in each system: in animal studies, phase-I and phase-II drug-metabolizing enzymes are operative, while in the Ames test the enzymes that utilize NADPH or NADH, such as quinone reductase and monooxygenases, are active. Differences between the results of the *in vivo* studies and the results of the Ames test may further be explained by species differences between mouse and rat (rat microsomes were used in the Ames test).

DNA adduct formation

In an earlier study with λ-*lac*Z-transgenic mice, we observed that the maximum adduct level in liver after i.p. injection of B[a]P (100 mg/kg b.w.) was reached after 2 days, with 40% still present after 42 days (unpublished data). In the present study the adduct levels were determined 48 days after treatment and these were similar to the adduct levels observed previously. The dietary treatment with eugenol had no effect on the total amount of adducts or the type of adducts that were formed by B[a]P, although small non-significant quantitative differences were observed.

A suitable candidate antimutagen should not be (geno)toxic *per se* [12]. Therefore, in order to exclude potential genotoxic effects of eugenol itself, the occurrence of

alkenylbenzene-DNA adducts was studied by ^{32}P-postlabelling. When liver DNA of mice treated with B[a]P was analyzed by ^{32}P-postlabelling with solvents suitable for chromatography of alkenylbenzene-DNA adducts, also 4 specific B[a]P-DNA adducts were detected. The total amount of B[a]P-DNA adducts that was observed in mice fed the eugenol diet was similar by both postlabelling methods (17.1 $\pm$ 4.4 adducts/10^8 nucleotides and 21.7 $\pm$ 6.3 adducts/10^8 nucleotides; $P > 0.05$). The total amount of B[a]P-DNA adducts determined by ^{32}P-postlabelling analysis with chromatographic conditions for B[a]P-DNA adducts were not significantly different between mice on the control diet and mice on the eugenol diet. However, the total amount of B[a]P-DNA adducts determined with the alkenylbenzene solvent system, was lower (although not significantly) in mice fed the eugenol diet than in mice fed the control diet (respectively, 21.7 $\pm$ 6.3 adducts/10^8 nucleotides and 33.4 $\pm$ 13.0 adducts/10^8 nucleotides). All individual adduct levels of B[a]P-DNA adducts were lower (although not significantly) in mice fed the eugenol diet than in mice fed the control diet. Although the differences in the amount of total and individual B[a]P-DNA adducts, determined with the alkenylbenzene solvent system, between mice on the eugenol diet and mice on the control diet are not statistically significant, which may be due to the small groups (n=4), this may still be biologically relevant. This may indicate some antigenotoxic potential of eugenol *in vivo*. Three of these adducts were also observed when standard BPDE-modified DNA was analyzed. In liver DNA from mice fed the eugenol diet another DNA adduct was observed. Based on earlier studies [19,20,28], in which no eugenol-DNA adducts could be detected, this was not expected. This finding will be further discussed elsewhere (Steenwinkel *et al.*, in preparation).

Phase-II biotransformation enzyme activities

In the present study phase-II enzyme activities were measured to determine whether eugenol-treatment leads to induction of phase-II enzymes in mice. In previous studies with eugenol, an induction of GST and GT activity was found in the liver of rats after treatment with eugenol for 10 days [6] or 13 weeks [26]. Zheng *et al.* [27] found a significant induction of total GST activity in the mouse liver (1.3-fold) and small intestine (2.4-fold) after oral administration of 20 mg eugenol per animal once every 2 days for a total of three administrations. The specific induction of phase-II biotransformation enzymes may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [7]. However, in the present study a significant inhibition of GST activity and no change in GT activity was found, after dietary treatment of mice with eugenol for 58 days. This may be due to strain differences, differences in route of administration and/or treatment time. In the present study, GST-inhibition did not result in higher mutant frequency or higher DNA-adduct levels induced by B[a]P in mice fed a diet containing 0.4% eugenol in comparison to mice fed the

control diet. This implicates that the remaining phase-II biotransformation capacity detoxifies B[a]P-metabolites to the same extent as in control mice. Another explanation may be an overall decrease in GST enzymes with no quantitative change in μ-class isoenzymes, which are most important for detoxification of polycyclic aromatic hydrocarbon-epoxides. GST-isoenzymes could not be determined because until now there is no technique available to separate and quantitate murine GST-isoenzymes.

The present study was performed to investigate whether eugenol, which has an antigenotoxic potential *in vitro*, also reveals this potential *in vivo*. It can be concluded that there is some indication that eugenol has an antigenotoxic potential against B[a]P *in vivo* in the mouse, while it cannot be excluded that eugenol *per se* is genotoxic *in vivo*.

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CHAPTER 7

EFFECT OF SHORT-TERM DIETARY ADMINISTRATION OF EUGENOL IN HUMANS

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Abstract

In order to study the antigenotoxic potential of eugenol in humans, ten healthy non-smoking males ingested a daily amount of 150 mg eugenol or the placebo for seven consecutive days. After a washout period of one week, groups ingesting eugenol or the placebo were crossed and received the other treatment for seven consecutive days.
On days 8 and 22 blood samples were taken for the assessment of standard clinical biochemical parameters. To study the possible antigenotoxic effect of eugenol, on day 8 and 22 blood samples were collected and exposed *in vitro* to the established genotoxic agents mitomycin C and vinblastine. After exposure the percentage of cells with chromosome aberrations and micronuclei was determined in cultured white blood cells. On days 8 and 22 paracetamol (500 mg p.o.) was administered as test substance to measure phase-II biotransformation capacity. Glutathione-S-transferase (GST) activities were determined in erythrocytes and blood plasma. No significant differences in the clinical biochemical parameters were detected between the eugenol-period and the placebo-period, indicating that daily administration of 150 mg eugenol for seven days has no toxic effects. No significant differences on the cytogenetic parameters were found after ingestion of eugenol. Thus, there are no indications for an antigenotoxic potential of eugenol in humans, consuming daily 150 mg eugenol for seven days. A significant reduction in α-

class GSTs in plasma ($P<0.05$), but not in the other measured biotransformation parameters, was found in volunteers during the eugenol-period as compared to the placebo-period. This may either reflect GST inhibition by eugenol or protection against background damage of liver cells by eugenol.

Introduction

Humans are exposed to a large variety of chemical substances from natural sources. In recent years it has become clear that naturally occurring compounds can have important effects on the consequences of exposure to carcinogens. Current scientific interest is directed towards the cancer-preventing potential of naturally occurring substances. Several groups of naturally occurring compounds, including flavonoids, indoles, aromatic isothiocyanates and terpenes, have been shown to possess antimutagenic and anticarcinogenic activities [1,2].

Alkenylbenzenes are an important group of naturally occurring food flavourings, among them eugenol (4-allyl-1-hydroxy-2-methoxybenzene). Eugenol is the main component of oil of cloves and is also present in the essential oils of many other plants, such as cinnamon, basil, and nutmeg. It has been used since at least the nineteenth century, primarely as a flavouring agent, in a variety of foods and pharmaceutical products, and as an analgesic in dental materials.

The Joint FAO/WHO Expert Committee on Food Additives established an acceptable daily intake (ADI) of eugenol of 0-2.5 mg/kg body weight for humans [3]. The daily *per capita* consumption is estimated to be 0.6 mg [4].

Recently, in a subacute study, we found a preferential induction of detoxifying phase-II biotransformation enzymes (glutathione S-transferase and glucuronyl transferase) by eugenol in rat liver *in vivo* [5], which may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [6]. Indeed for eugenol there are indications for protective effects against chemically induced mutagenesis *in vitro* and *in vivo*. Eugenol inhibited the mutagenicity of aflatoxin B_1 and N-methyl-N'-nitro-N-nitrosoguanidine in *Salmonella typhimurium* tester strain TA100 [7]. In addition, microsomes or S9 prepared from rats that received eugenol decreased the mutagenic activity of benzo[a]pyrene in the Ames test in comparison to the microsomes or S9 from untreated rats [8,9]. In the rodent bone marrow micronucleus test using male Swiss mice, oral administration of eugenol (0.4% in the diet) for 15 days was found to decrease significantly the frequency of micronucleated polychromatic erythrocytes elevated by cyclophosphamide [10]. Whether these *in vitro* and *in vivo* findings with high doses of eugenol implicate a dietary cancer

preventive effect of eugenol in humans at low dose remains to be investigated. Therefore a short-term intervention study in human volunteers has been performed to study the effect of subacute oral intake of eugenol on clinical parameters, phase-II biotransformation capacity and *in vitro* exposure to established genotoxic agents.

Materials and methods

Materials

Eugenol (CAS Reg. No. 97-53-0; food-grade), vinblastine and mitomycin C were obtained from Sigma Chemical Company (St. Louis, USA). RPMI 1640 medium was purchased from Gibco BRL, Life Technologies (Paisley, Scotland). Gentamicin was obtained from Gibco BRL (Breda, The Netherlands) and foetal bovine serum from Integro B.V. (Zaandam, The Netherlands). Phytohaemagglutinin HA 15 (PHA) was obtained from Murex Diagnostics Ltd (Dartford, England) and cytochalasin B from Aldrich Chemie (Brussels, Belgium). For each participant 23 eugenol capsules (including 1 spare capsule) containing each 50 mg eugenol (dissolved in dried starch) and 23 placebo capsules (including 1 spare capsule) containing dried starch were made within 1 week before the beginning of the study.

Experimental protocol

Formal approvement by the TNO Medical Ethical Committee was obtained prior to the start of the selection of volunteers. Ten healthy male volunteers (21-26 years of age, body weight 68-88 kg) gave their informed consent and participated in the trial. Their liver and kidney functions were normal according to the appropriate clinical biochemical parameters. All participants were non-smokers and had to follow few dietary restrictions during the study: no cloves, cinnamon, basil, nutmeg, roasted/grilled meat, Brussels sprouts or vitamin preparations. The volunteers were not allowed to take paracetamol (acetaminophen) on day 7 and 21 of the study. They were at random allocated to one of two groups. In the first week one group (n=5) received capsules containing 50 mg eugenol and the other group (n=5) received the placebo. Capsules were reached out in a pill bottle containing 3 drops of *menthe piperitea aeteroleum* in order to cover up the fragrance of eugenol. The participants were instructed to take 1 capsule three times a day (8.00, 16.00 and 23.00 h). In the second week none of the groups ingested capsules (wash-out period). In the third week the two groups were exchanged and received the other treatment (1 capsule containg a placebo or 50 mg eugenol, three times a day). There were no drop-outs. A detailed survey of the experimental protocol is presented in Table 1.

Table 1. Day to day protocol for the determination of the effect of oral intake of eugenol (150 mg/day for 7 days) by ten male volunteers on clinical biochemical parameters, phase-II biotransformation enzyme activities and cytogenetic parameters.

day	eugenol-intake		number of capsules/day[a]	blood sampling[b]	PC-test[c]
	group I (n=5)	group II (n=5)			
1	eugenol	placebo	3		
2	eugenol	placebo	3		
3	eugenol	placebo	3		
4	eugenol	placebo	3		
5	eugenol	placebo	3		
6	eugenol	placebo	3		
7	eugenol	placebo	3		
8	eugenol	placebo	1	+	+
9					
10					
11					
12					
13					
14					
15	placebo	eugenol	3		
16	placebo	eugenol	3		
17	placebo	eugenol	3		
18	placebo	eugenol	3		
19	placebo	eugenol	3		
20	placebo	eugenol	3		
21	placebo	eugenol	3		
22	placebo	eugenol	1	+	+

+ = performed

[a] capsules (containing 50 mg eugenol or the solvent control) were ingested at 8.00 a.m., 16.00 p.m. and 23.00 p.m. with exception of day 8 and 22: 1 capsule at 8.00 a.m.

[b] 12 ml heparinized blood was sampled for determination of glutathione S-transferase levels (in erythrocytes and plasma) and cytogenetic parameters; 5 ml cloth blood and 5 ml EDTA-blood were sampled for clinical biochemical parameters.

[c] Volunteers ingested paracetamol (500 mg, p.o.) at 8.30 a.m. After 5 h EDTA-blood (5 ml) was sampled.

Clinical biochemical parameters

For the determination of clinical biochemical parameters, cloth blood and EDTA-blood were sampled on day 8 and 22. In EDTA-blood samples, numbers of leucocytes and platelets, haemoglobin content and haematocrit were determined. Blood cells were counted by electrical resistance detection on a Sysmex K-1000 haematology analyser. Levels of L-aspartate aminotransferase (ASAT), L-alanine aminotransferase (ALAT),

γ-glutamyl transpeptidase, lactate dehydrogenase and alkaline phophatase were determined in plasma using a Cobas-Bio centrifugal analyser.

Phase-II biotransformation enzyme acticities

For the determination of GST levels in erythrocytes and plasma, heparinized blood samples were collected on day 8 and 22. Aliquots of 100 μl heparinized blood were added to 2 ml physiologic salt solution and washed three times. The pellet was haemolysed in 0.9 ml Nonidet solution and stored at -80°C. The remaining heparinized blood was centrifuged and the plasma samples were stored at -80°C. The activity of GST in haemolysed erythrocytes was determined with 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* [11]. α-Class GSTs were measured in 100 μl plasma by radioimmunoassay with delayed tracer addition as described by Bogaards *et al.* [12].

The paracetamol test followed the protocol of Miners *et al.* [13]. In short, after fasting overnight, volunteers ingested 500 mg paracetamol with a glass of mineral water on days 8 and 22. After 5 h blood samples were collected in tubes containing EDTA. After centrifugation, plasma samples were stored at -20°C until analysis. The method for the measurement of plasma levels of paracetamol was slightly modified from Verhagen *et al.* [14]. A 0.6 ml aliquot of plasma was mixed with 0.6 ml of 20% trichloroacetic acid. After centrifugation, 20 μl of the clear supernatant was injected onto a HPLC column. The HPLC system consisted of a Zorbax ODS column (4.6 mm x 25 cm), a 2140 Rapid Spectral Detector (Pharmacia, Uppsala, Sweden) operating at 254 nm, using a gradient of water/acetonitril/acetic acid (94.5/4.5/1; solvent A) and 100% acetonitril (solvent B). The gradient consisted of a linear gradient from 0 to 35% solvent B in 10 min. Flow was 0.9 ml/min; paracetamol eluted after 8.3 min. Plasma clearance of paracetamol was calculated as desribed by Døssing *et al.* [15,16].

Cytogenetic parameters

Aliquots (10 ml) of blood were collected in heparinized tubes from each of the volunteers on day 8 and 22. 0.5 ml-Aliquots of whole blood were added to 4.5 ml prewarmed RPMI 1640 medium, supplemented with 50 μg/ml gentamicin.

For the assessment of micronucleated-binucleates, duplicate cultures were treated with vinblastine (0.05 μg/ml) or mitomycin C (0.3 μg/ml or 0.5 μg/ml). Control cultures were exposed to the solvent control (saline) only. After 2 hours incubation at 37°C under 5% CO_2 in air at 100% relative humidity, blood cultures were washed with medium. Pellets were resuspended in 4.5 ml of RPMI 1640 medium supplemented with 20% foetal bovine serum, 225 μg/ml PHA and 50 μg/ml gentamicin. After 45 h incubation at 37°C, cytochalasin B was added at 3 μg/ml (final concentration). At 75 h after the initiation of

the cultures cell preparations were made following hypotonic treatment with 0.075 M KCl at room temperature for 5 min, fixation in methanol/acetic acid (3/1, v/v for all but the last fixation step, 9/1 for the last fixation step), spreading onto glass slides and air-drying. Slides were stained for 15 min with 2% Giemsa, air-dried and embedded in Depex. All glass slides were coded prior to analysis. Per treatment a total of 500 binucleated lymphocytes (from two slides) was scored for the incidence of micronucleated binucleates.

For the assessment of chromosome aberrations, duplicate cultures were treated with mitomycin C (0.3 μg/ml or 0.5 μg/ml). After 2 hours incubation at 37°C under 5% CO_2 in air at 100% relative humidity, blood cultures were washed with medium. Pellets were resuspended in 4.5 ml of RPMI 1640 medium supplemented with 20% foetal bovine serum, 225 μg/ml PHA and 50 μg/ml gentamicin. After 46 h incubation at 37°C, 0.1 μg/ml colcemid was added. At 50 h after the initiation of the cultures cell preparations were made following hypotonic treatment with 0.075 M KCl at 37°C for 15 min, fixation in methanol/acetic acid (3/1, v/v), spreading onto glass slides and air-drying. Slides were stained for 10 min with 2% Giemsa, air-dried and embedded in Depex. All glass slides were coded prior to analysis. Per treatment a total of 50 metaphases (from two slides) was scored for the incidence of chromosome aberrations.

Compliance of eugenol-intake determined in 24 h-urine by proton NMR spectroscopy

On day 7 and 21 the volunteers collected urine after the first morning urine up to and including the urine of the next morning. A volume of each sample was adjusted to pH 7.0 with 1 M NaOH and lyophilised. Of the remaining substance 100 mg was taken and reconstituted in deuteriumoxide. After filtration each urine was placed in a 5 mm o.d. NMR tube and a ^{1}H-NMR spectrum was recorded on a Varian UNITY-400 FT NMR spectrometer operating at a proton NMR frequency of 400 MHz at 30°C using a fully automated ASM100 sample changer. A typical NMR spectrum consisted of 128 transients using 65536 digital points over a 8000 Hz bandwidth using a 9.8 μs (45°) rf pulse. Total acquisition time of a spectrum was 21 min.

Statistical design and analysis

For the experiment, a cross-over design was used [17]. This design is such that differences between subjects, and an overall difference between the periods, do not affect the comparison of the treatments. The data were evaluated with analysis of variance accounting for differences between subjects, between periods, and between treatments, respectively. The analysis is exemplified by Cochran and Cox [17]. In the presentation of the results, the standard deviation is replaced by the least significant difference at the 5%

level (LSD). This is because the standard deviation as calculated in the usual way contains variation between subjects and between periods, while comparison of the treatments should be made on a within-subject-within-days basis.

Results

Clinical biochemical parameters

Administration of eugenol had no effect on the clinical biochemical parameters measured (Table 2). All values fell within the range of control values.

Table 2. Clinical biochemical parameters of male volunteers treated with eugenol.

Treatment	HGB (mM)	HCT (litre/ litre)	WBC (x 10^9/ litre)	PLT (x 10^9/ litre)	ASAT (U/litre plasma)	ALAT (U/litre plasma)	γ-GT (U/litre plasma)	LDH (U/litre plasma)	AP (U/litre plasma)
Placebo	9.8	0.4	5.5	232	21.7	19.3	17.4	331	79.0
Eugenol	9.6	0.4	5.7	236	23.0	19.0	17.7	333	78.2
	(0.2)	(0.0)	(0.6)	(17)	(4.8)	(3.9)	(3.5)	(32)	(4.1)

Haemoglobin content (HGB), haematocrit (HCT), numbers of leucocytes (WBC) and platelets (PLT), L-aspartate aminotransferase (ASAT), L-alanine aminotransferase (ALAT), γ-glutamyl transpeptidase (γ-GT), lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activities were measured in blood samples of men (n=10) orally treated with eugenol (3 times a day 50 mg) for 7 consecutive days. Blood samples were collected 8 h after the last dose. Values are means. The least significant difference (5%) is given in parentheses.

Phase-II enzyme activities

No effect of eugenol was found on the clearance of paracetamol and on the GST activity in erythrocytes (Table 3). In contrast, administration of eugenol caused a significant decrease of 26% in levels of α-class GSTs in plasma ($P<0.05$).

Cytogenetic parameters

The established mutagens mitomycin C and vinblastine significantly induced the incidence of micronucleated binucleates *in vitro* as compared to the solvent control saline; administration of eugenol had no effect on the incidence of micronucleated binucleates induced *in vitro* by mitomycin C and vinblastine (Fig. 1).

Table 3. Phase-II biotransformation activities in blood samples of male volunteers treated with eugenol.

Treatment	GST (U/litre blood)	GST-α (μg/litre plasma)	Cl_{PC} (ml/min)
Placebo	619	5.7	473
Eugenol	611	4.2*	496
	(37)	(1.3)	(30)

Glutathione S-transferase (GST) activity in erythrocytes and levels of α-class GSTs (GST-α) in plasma of male volunteers (n=10) who ingested eugenol (3 times a day 50 mg) or a placebo for 7 consecutive days. Clearance of paracetamol (Cl_{PC}) determined in male volunteers, who ingested 500 mg paracetamol immediately after ingestion of eugenol (3 times a day 50 mg) or of a placebo for 7 consecutive days. All values are means. The least significant difference (5%) is given in parentheses. Asterisk indicates significant difference from placebo-group ($P<0.05$).

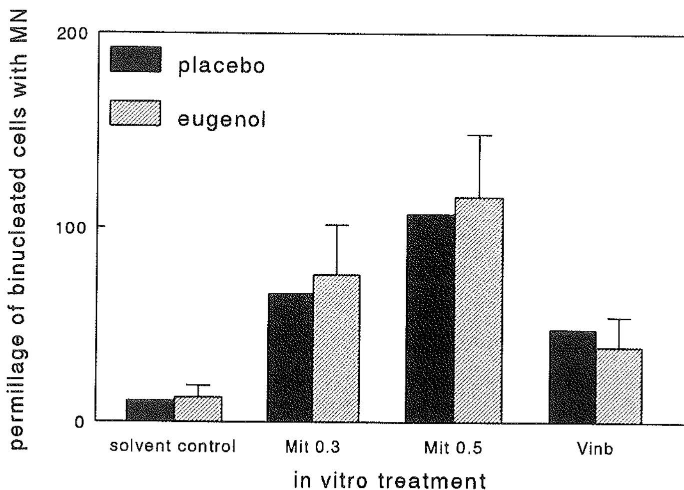

Fig. 1. Permillage of binucleated lymphocytes with micronuclei (MN) (500 cells scored) in blood samples of male volunteers (n=10) who ingested eugenol (3 times a day 50 mg) or a placebo for 7 consecutive days; cells were exposed *in vitro* to vinblastine (0.05 μg/ml) or mitomycin C (0.3 or 0.5 μg/ml) for 2 h prior to culturing. Values are means $\pm$ LSD (5%).

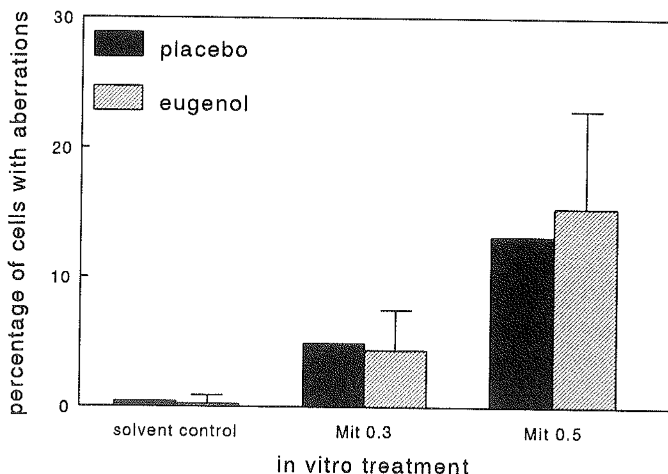

Fig. 2. Percentage of lymphocytes with chromosome aberrations excluding gaps (50 cells scored) in blood samples of male volunteers (n=10) who ingested eugenol (3 times a day 50 mg) or a placebo for 7 consecutive days; cells were exposed *in vitro* to 0.3 or 0.5 μg/ml mitomycin C for 2 h prior to culturing. Values are means ± LSD (5%).

Mitomycin C significantly induced the percentage of lymphocytes with chromosome aberrations *in vitro* as compared to the solvent control saline; administration of eugenol had no effect on the percentage of lymphocytes with chromosome aberrations induced *in vitro* by mitomycin C (Fig. 2).

Compliance of eugenol-intake

In all 24-h urines of volunteers consuming eugenol, eugenol could be detected in the proton NMR spectra (Fig. 3). In the 24-h urines of volunteers consuming a placebo, no eugenol was detected (Fig. 3).

Discussion

Seven days of oral administration of 150 mg eugenol (80% of ADI) to humans was not expected to induce toxic effects. Indeed, no significant differences in the clinical biochemical parameters between eugenol-treatment and placebo-treatment were detected.

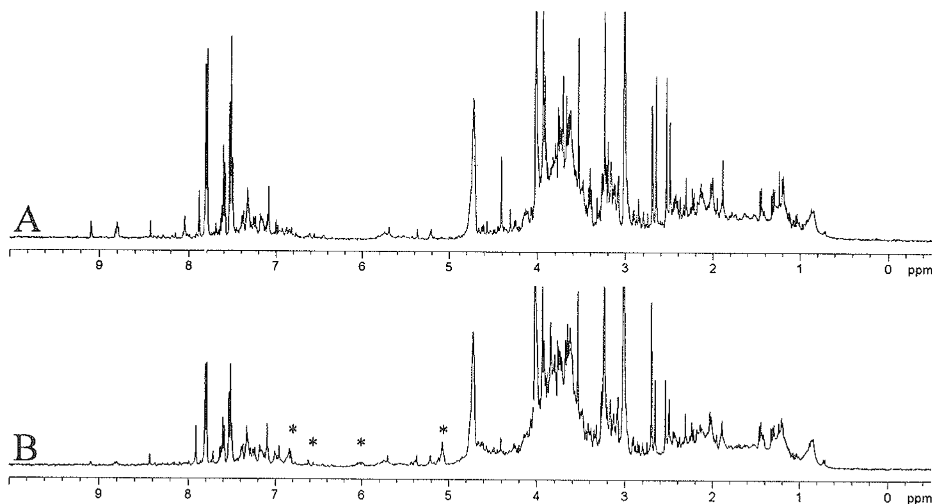

Fig. 3. Proton NMR spectra of (A) 24-h urine of a volunteer consuming a placebo and (B) 24-h urine of a volunteer consuming 150 mg eugenol. Asterisks indicate peaks of eugenol.

In previous studies with experimental animals, an induction of GST and glucuronyl transferase activity was found in the liver of rats after treatment with eugenol for 10 days or 13 weeks [5,18]. Zheng *et al.* found a significant induction of total GST activity in the mouse liver (1.3-fold) and small intestine (2.4-fold) after oral administration of 20 mg eugenol per animal once every 2 days for a total of three administrations [19]. The preferential induction of phase-II biotransformation enzymes may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [6].

Paracetamol is a well established test substance for the assessment of phase-II biotransformation capacity in man [13,14]. Paracetamol is mainly cleared from plasma by conjugation with glucuronic acid or sulphate, followed by renal excretion. Under therapeutical conditions for paracetamol only about 15% of the dose is metabolized via other routes or excreted unmetabolized [13]. In the present study, no significant differences in paracetamol clearance were found in the paracetamol test, indicating that oral intake of eugenol at 80% of the ADI for 7 days does not modulate glucuronidation and sulphation capacity in humans. In addition, also no significant difference in GST activity in the erythrocytes was found. In contrast, a significant reduction of α-class GSTs after dietary treatment with eugenol for 7 days was found. In the human liver, at least 75% of the GSTs consists of α-class GSTs [20]. Therefore, α-class GSTs are quantitatively

important for the detoxification of many compounds via human liver, and induction of α-class GSTs (e.g. in the liver) by food constituents may be a mechanism underlying their protective capacity against carcinogenic compounds [12]. α-Class GSTs are released into the plasma when hepatic damage occurs, and measurement of α-class GSTs in plasma is used for detection of hepatocellular damage. It is a more accurate and sensitive index than ASAT and ALAT because α-class GSTs are distributed uniformly across the liver lobule (and thus reflects the damage of the whole liver), whereas ASAT and ALAT are found predominantly in the periportal hepatocytes [21,22,23]. However, since turnover of liver cells also occurs under non-hepatotoxic conditions, measurement of GST levels in plasma may be used as a biomarker of the whole liver GST concentration [12]. In addition to the liver, other organs, such as the small intestine, also contain α-class GST and turnover of these cells will also contribute to the α-class GST levels in plasma [24,25,26,27]. The findings in the present study indicate that daily dietary intake of eugenol at 80% of the ADI for 7 days leads to a decrease of α-class GST levels in plasma. Since these findings were obtained in the absence of any adverse effects this decrease may reflect an enzyme decrease of α-class GSTs in liver (and other tissue) which may be due to GST inhibition by eugenol and which implicates that eugenol may be a candidate compound to overcome the resistance to chemotherapeutic drugs in cancer tissues caused by GST [28]. This is in agreement with the results of a recent study with mice in which a 50% inhibition of total GST activity was found in liver of mice, fed a diet containing 0.4% eugenol for 58 days [29]. Recently, the effect of eugenol on GST activity was further studied *in vitro*, and clear species differences in GST inhibition were found using cytosol of rats, mice and humans. The rate of inhibition of GST by eugenol (in the presence of tyrosinase) was highest in mouse cytosol and lowest in rat cytosol [30]. Another explanation for the present finding on reduction of α-class GST levels in plasma, may be that eugenol protects against background damage of liver cells (and other cells) and thus leads to less release of α-class GST in plasma by inhibition of lipid peroxidation. As such, it has been shown that eugenol protects against lipid peroxidation [31,32,33].

It is important that an established antigenotoxic response in *in vitro* test systems should be verified in *in vivo* test systems [34]. If no antigenotoxic potential *in vivo* is evident a classification as antigenotoxic substance is no more appropriate [35]. Beyond these experimental studies, the most valuable data on chemopreventive effects of dietary compounds towards humans come from studies in humans. Eugenol has been shown to possess an antigenotox potential *in vitro* and *in vivo* in experimental animals [7,8,9,10, 36]. Therefore it is relevant to study the antigenotoxic potential of eugenol in man. For this purpose cytogenetic biomarkers (micronuclei and chromosome aberrations) are suitable. Eugenol itself had no effect on the background level of micronucleated binuclea-

tes and the percentage of cells with chromosome aberrations, indicating that eugenol was not genotoxic in humans, as expected. However, no effect of eugenol on the incidence of micronucleated binucleates induced by mitomycin C and vinblastine and the percentage of cells with chromosome aberrations induced by mitomycin C was found.

In summary, it can be concluded that consumption of eugenol for seven days at 80% of the ADI has no adverse effects in humans. Furthermore, there are no indications for an induction of phase-II enzyme activities and for an antigenotoxic potential of eugenol in man. In contrast, a significant decrease in levels of GST-α in plasma of humans, who consumed eugenol for 7 days, was found indicating that α-class GSTs in liver may be decreased due to protection against background damage of liver cells by eugenol or due to GST inhibition by eugenol.

Acknowledgements

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CHAPTER 8

INHIBITION OF RAT, MOUSE AND HUMAN GLUTATHIONE S-TRANSFERASE BY EUGENOL AND ITS OXIDATION PRODUCTS

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Abstract

The irreversible and reversible inhibition of glutathione S-transferases (GSTs) by eugenol was studied in rat, mouse and man. Using liver cytosol of human, rat and mouse, species differences were found in the rate of irreversible inhibition of GSTs by eugenol in the presence of the enzyme tyrosinase. Tyrosinase was used to oxidize eugenol. No inhibition was observed in the absence of tyrosinase. The rate of irreversible inhibition of GSTs was highest in mouse cytosol, and lowest in rat cytosol. In addition, the irreversible inhibition of human and rat GSTs by eugenol was studied using purified isoenzymes of man and rat. The human GST isoenzymes A1-1, M1a-1a and P1-1 and the rat GST isoenzymes 1-1, 2-2, 3-3, 4-4 and 7-7 were irreversibly inhibited by eugenol in the presence of tyrosinase. In this respect human GST P1-1 and rat GST 7-7 were by far the most sensitive enzymes; human GST A2-2 was not inhibited. Indications were found that human GST P1-1 may be inhibited via three mechanisms: in addition to the well documentated nucleophilic addition of quinones and oxidation of cysteine residues, also a covalent subunit cross-linking was observed. The reversible inhibition of human and rat GST by eugenol, eugenol methyl ether, isoeugenol methyl ether, 2-allylphenol and 4-propylphenol was also studied using purified isoenzymes. The reversible inhibition of human and rat GSTs, using 1-chloro-2,4-dinitrobenzene as substrate was expressed as I_{25}.

All compounds caused moderate reversible inhibition (I_{25} ranged from 0.2-5.4 mM for human GSTs and from 0.4-4.9 mM for rat GSTs). In rat, eugenol methyl ether was the strongest inhibitor. In human, the overall inhibiting capacities of eugenol, eugenol methyl ether, isoeugenol methyl ether and 4-propyl phenol were more or less similar; 2-allylphenol was the poorest inhibitor.

Introduction

Eugenol, a naturally occurring phenolic compound, is a major component of clove oil and is also present in oils of cinnamon, basil and nutmeg (Figure 1). It is generally recognized as a safe food additive (GRAS-status) and is mainly used as a flavouring agent in foods such as baked products, beverages, sweets and frozen dairy products [1,2]. Eugenol has been shown to be an antimutagenic compound *in vitro*. It inhibited the mutagenicity of aflatoxin B_1, N-methyl-N'-nitro-N-nitrosoguanidine and dimethylbenzanthracene in the Ames test [3,4]. In addition, microsomes or S9 prepared from rats that received eugenol decreased the mutagenic activity of benzo[a]pyrene in the Ames test in comparison to the microsomes or S9 from untreated rats [5,6]. There are also indications for an antigenotoxic potential of eugenol *in vivo*. In the rodent bone marrow micronucleus test using male Swiss mice, oral administration of eugenol (0.4% in the diet) for 15 days was found to decrease significantly the frequency of micronucleated polychromatic erythrocytes elevated by cyclophosphamide [7].

Besides these antigenotoxic properties of eugenol, there is evidence that eugenol forms a quinone methide species by one- or two-electron oxidative pathways that is potentially capable of alkylating cellular proteins and thiols. One pathway is a two-electron oxidation of eugenol catalyzed by cytochrome P-450. Alternatively, quinone methide formation can occur through the intermediate formation of a phenoxy radical, which either disproportionates or undergoes further oxidation to yield the quinone methide [8]. Tsai *et al.* [9] postulated that eugenol may form a quinone methide from its carbonium ion by deprotonation. Indirect experimental proof exists for the formation of the quinone methide as an intermediate in the metabolism of eugenol, since the oxidation of this compound with liver or rat lung microsomes yielded glutathione conjugates at both the benzylic and ω-carbon atoms [10]. However, little is known of the actual interaction of eugenol with nucleic acids and cellular proteins. Recently, in liver DNA of mice fed a diet containing 0.4% eugenol for 58 days, an eugenol-induced DNA-adduct was detected [11,12], which could result from DNA-alkylation by the quinone methide of eugenol.

Glutathione S-transferases (GSTs) are sensitive to an irreversible inactivation by quinones as a result of covalent modification [13]. Induction of GSTs is usually assumed to result in decreased cancer risk [14,15]. Recently, in a subacute study, we found an induction of glutathione S-transferase activity by eugenol in rat liver *in vivo* [16], which may be an indication that eugenol possesses antimutagenic/anticarcinogenic properties [17]. In contrast, inhibition of GST activity was found in liver of eugenol-treated mice [12], while in humans, consumption of 150 mg eugenol for 7 days resulted in a 26% decrease in α-class GSTs in plasma [18]. This prompted us to investigate the irreversible and reversible inhibitory characteristics of eugenol in different species. The irreversible inhibition of eugenol itself and of its oxidation products on GST was studied. To study the effect of chemical characteristics of eugenol involved in the reversible inhibition, the

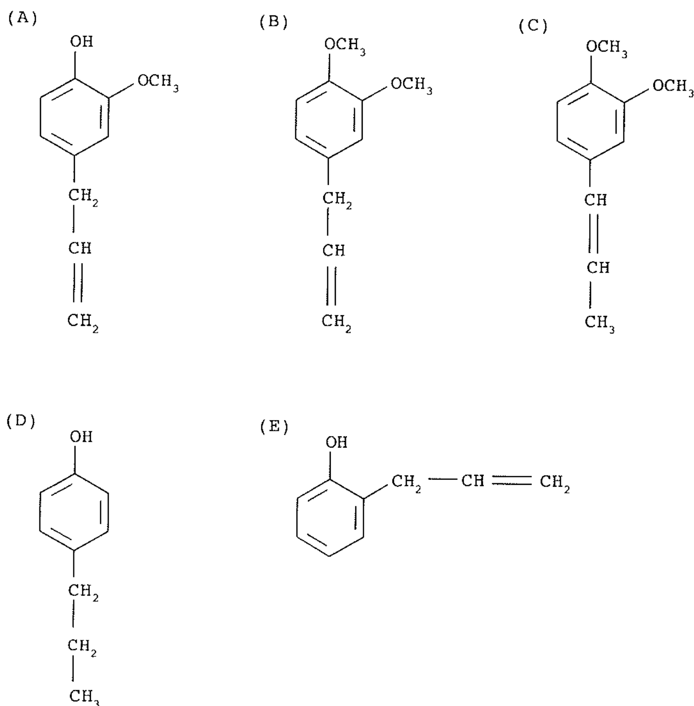


Fig. 1. The molecular structure of eugenol (A), eugenol methyl ether (B), isoeugenol methyl ether (C), 4-propylphenol (D) and 2-allylphenol (E).

reversible GST inhibition by compounds structurally related to eugenol was determined, namely 2-allylphenol, 4-propylphenol, eugenol methyl ether and isoeugenol methyl ether (Fig. 1).

Materials and methods

Chemicals

Eugenol (CAS No. 97-53-0; purity 99%) was obtained from Janssen Chimica (Tilburg, The Netherlands). 2-Allylphenol (CAS No. 1745-81-9; purity 98%) and 4-propylphenol (CAS No. 645-56-7; purity 99%) were obtained from Aldrich-Chemie (Steinheim, Germany). Eugenol methyl ether (CAS No. 93-15-2; purity 98%) and isoeugenol methyl ether (CAS No. 93-16-3; purity 98%) were obtained from ICN Biochemicals, Inc. (Cleveland, Ohio, USA). Glutathione was from Boehringer (Mannheim, Germany). Tyrosinase (from mushroom; 3130 units/mg solid; E.C. 1.14.18.1) was from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of human, rat and mouse liver cytosol

Liver cytosol from rat (male Wistar rats from Charles River Wiga, Sulzfeld, Germany) and mouse (male λ-*lacZ*-transgenic mouse strain 40.6, a derivative of (BALB/c x DBA/2)CD2F1, from TNO Centre for Animal Research, Rijswijk, The Netherlands) was prepared as described previously [6,12]. Human liver cytosol was prepared by homogenizing liver (obtained at autopsy from kidney donors or from surgical biopsies) with 3 volumes of 0.01 M Tris-HCl/0.14 M KCl pH 7.4 with a Potter Elvehjem tissue homogenizer and centrifuging at 105,000 x *g* for 75 min at 4°C. The cytosol was stored at -30°C. Cytosol was pooled (human n=4; rat n=3; mouse n=3) and half of the cytosol (0.6-1.5 ml) was dialyzed overnight against 5 liters of 1 mM Tris/10 mM KCl pH 7.4 at 4°C.

Purification of human and rat glutathione S-transferase isoenzymes

GST isoenzymes were purified from rat liver and kidney (GST 7-7) and human liver and placenta (human GST P1-1) using affinity chromatography (S-hexylglutathione-Sepharose 6B) as described by Vos *et al.* [19]. Separation of the various isoenzymes was achieved by chromatofocusing as described previously [20]. Purity was confirmed by SDS gel electrophoresis, isoelectric focussing and HPLC-analysis [21]. Protein concentration was quantified by the method of Lowry *et al.* [22], using bovine serum albumin as standard.

Glutathione S-transferase assay

The activity of cytosolic or individual GST isoenzymes was determined with 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as second substrate at 25°C in 0.1 M potassium phosphate buffer pH 6.5 supplemented with 1 mM EDTA, using the spectrophotometric method of Habig *et al.* [23].

Tyrosinase-catalyzed oxidation of eugenol

To identify whether tyrosinase may produce a quinone methide-like intermediate as published by Thompson *et al.* [8], eugenol (1 mM) was incubated with 3 mM glutathione and 100 U/ml tyrosinase in 0.05 M potassium phosphate buffer pH 7.4 (final volume 1 ml). After 30 min of incubation at room temperature the solution was lyophilized and redissolved in water to a concentration of 10-20 pmol/μl for glutathione. The masses of the oxidation products of eugenol were determined by matrix-assisted laser desorption/ionization mass spectrometry using a VISION 2000 reflector-type time-of-flight laser desorption instrument (Finnigan MAT, Bremen, Germany) equipped with a nitrogen laser at 337 nm. The ions were accelerated to a potential of 6.5 kV in the ion source and post-accelerated by a conversion dynode in front of the detector to a potential of 20 kV. The effective drift length of the instrument is 1.7 m. Ions were detected by a secondary electron multiplier and the signal was amplified and digitized by a high-speed transient recorder. The matrix used was a 9:1 mixture of 10 mg/ml 2,5-dihydroxybenzoic acid in 0.1% (v/v) trifluoro acetic acid (TFA) with 10 mg/ml 2-hydroxy-5-methoxybenzoic acid in ethanol. Sample solutions with a typical concentration of 10-20 pmol/μl in 0.1% (v/v) TFA were mixed in the matrix solution (1/1, v/v). One μl of the matrix/sample solution was applied to a stainless-steel target, air-dried, and introduced into the mass spectrometer.

Irreversible inhibition studies

The time-dependent irreversible inhibition of cytosolic GST (undialysed and dialysed) was measured by incubating pooled human, rat or mouse cytosol (final protein concentration, respectively 97, 236, 103 μg/ml in incubations using undialysed cytosol and 41, 182 and 49 μg/ml in incubations using dialysed cytosol) with 100 μM eugenol in the presence of 100 units/ml tyrosinase (with and without further addition of 1 mM glutathione) at room temperature in 0.05 M potassium phosphate buffer pH 7.4 (final volume 200 μl). After 15, 30, 60, 120 and 240 min of incubation, a 10 μl sample was transferred in a microcuvette (total volume 250 μl) and the catalytic activity was determined towards CDNB according to Habig *et al.* [23]. Remaining activity was expressed as percentage of the corresponding blank incubation (5% ethanol).

The time-dependent irreversible inhibition of GST isoenzyme was measured under the same conditions as above by incubating 0.5 μM purified human or rat GST isoenzymes (final volume 200 μl), with the exception that the catalytic activity was determined after 30 min of incubation.

Analysis of the GST-adduct formation by mass spectrometry

A 0.5-ml solution (pH 7.4) of 10 μM GST P1-1 or 5 μM M1-1A and a 1.0-ml solution of 10 μM A1-1 were incubated with 100 μM eugenol at 25°C for 1 h. The reaction was stopped by placing on ice; ascorbic acid was added to a final concentration of 10 mM to avoid further massive oxidation. The solutions were extensively dialyzed (three times against three changes of 1.1 liter of 0.1% (v/v) solution of formic acid) and subsequently lyophilized. The masses of enzymes were determined by matrix-assisted laser desorption/ionization mass spectrometry as described above.

Reversible inhibition studies

The reversible inhibition was determined by mixing in the cuvettes of the enzymic assay (in final concentrations) 10-30 nM purified human- or rat GST isoenzymes with 0.08, 0.31, 0.63, 1.25, 2.50 and 5.00 mM of eugenol, 0.08, 0.31, 0.63, 1.25 and 2.50 mM of eugenol methyl ether, 0.10, 0.50, 1.25, 1.88 and 2.50 mM of isoeugenol methyl ether, 0.10, 0.50, 2.50, 5.00 and 12.50 mM of 2-allyl phenol or 0.07, 0.33, 1.64, 3.28 and 8.21 mM of 4-propyl phenol, after which the enzymatic activity was measured immediately as described by Habig *et al.* [23].

Results

Identification of oxidation product of eugenol

To identify whether in the presence of tyrosinase eugenol is oxidized to a quinone methide, which subsequently conjugates with glutathione, the mass of the resulting conjugate was determined. A mass of 510.0 was measured which is equivalent with the M^+ peak of the mono-glutathionyl conjugate of eugenol. In the spectrum, 5 additional unknown peaks were observed with masses of 653.8, 692.5, 730.8, 769.8 and 808.3 (results not shown).

Irreversible inhibition by cytosolic GST

The time-course of GST inhibition with eugenol for (undialysed) human, rat and mouse cytosol is shown in Figure 2. In all cases eugenol itself caused no irreversible inhi-

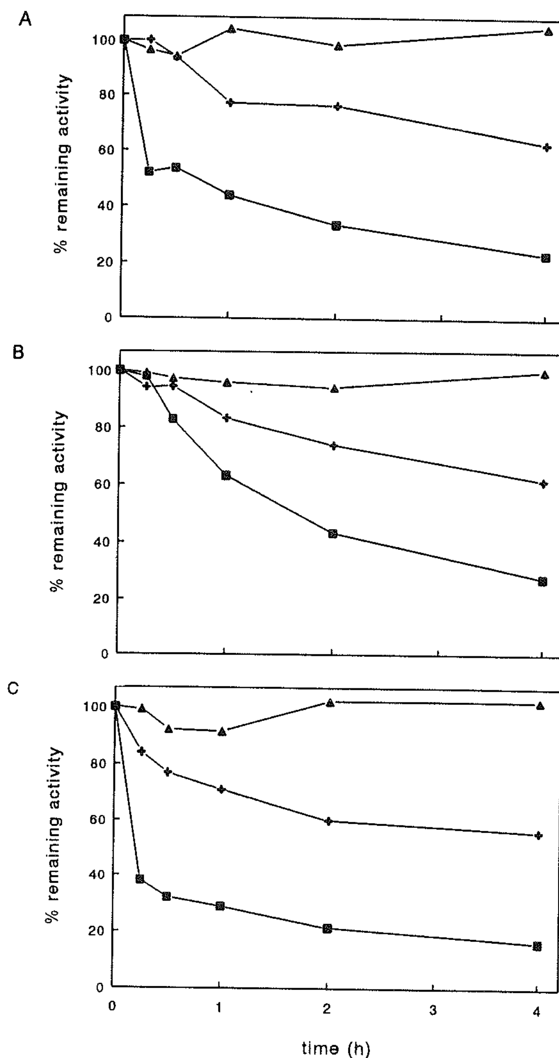


Fig. 2. Time-dependent irreversible inhibition of cytosolic GST (undialysed) by eugenol in the absence and presence of tyrosinase and glutathione (▲ = eugenol; ■ = eugenol with tyrosinase; + = eugenol with tyrosinase and glutathione). (A) Human- , (B) rat- or (C) mouse cytosol (final protein concentration, respectively 97, 236, 103 μg/ml) was incubated with 100 μM eugenol in the absence/presence of 100 units/ml tyrosinase (with and without further addition of 1 mM glutathione) at room temperature. Blanc incubations were performed in the same way, using ethanol as solvent control. At various time intervals, the catalytic activity was determined towards CDNB. Values are the average of two incubations and expressed as % remaining activity of blanc incubation ($\pm$ SD). For experimental details see Materials and methods. Percentage variationcoefficient was always less than 20% for individual values.

bition of cytosolic GST, but after addition of tyrosinase the cytosolic GST was inhibited. By addition of glutathione the irreversible inhibition could be partly slowed down. The same results were found with dialysed cytosol of human, rat and mouse (results not shown). A clear species difference was found in the rate of GST inhibition by eugenol in the presence of tyrosinase: with 30 min incubation, a remaining activity of 83, 54 and 32% as compared to their controls, was found using rat, human and mouse cytosol, respectively. After 4 h of incubation the GST cytosols of rat, human and mouse reached almost similar values: they were inhibited to 28, 23 and 16% remaining activity, respectively.

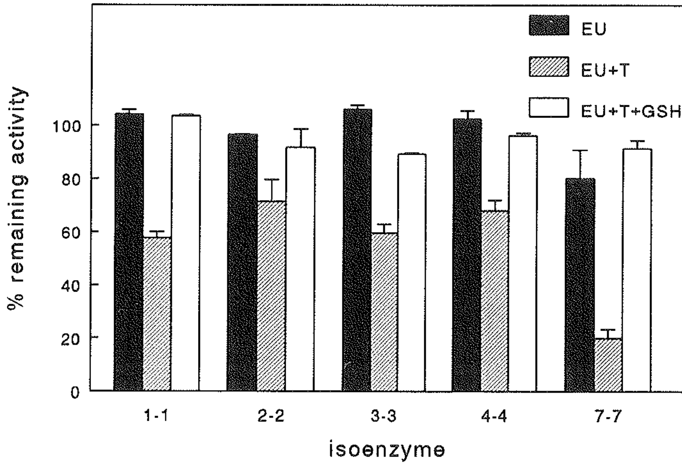
Irreversible inhibition of purified GST isoenzymes

The irreversible inhibition of human and rat GSTs by eugenol is shown in Figure 3. Eugenol itself caused no irreversible inhibition of GST isoenzymes of man and rat, whereas addition of tyrosinase, to generate a quinone methide, caused a clear inhibition of all used human and rat isoenzymes, except human GST A2-2. GST isoenzymes of the π-class are by far the most sensitive isoenzymes, in both man and rat. Again, glutathione protected against irreversible inhibition.

Analysis of irreversible inhibition of purified GST isoenzymes by mass spectrometry

Figure 4a shows the matrix-assisted laser desorption/ionization (MALDI) spectrum of GST P1-1. As in most cases for proteins with a number of equal non-covalently bound subunits, the spectrum only shows the M^{2+}, M^+ and $2M^+$ ions for the dissociated protein subunits [24,25]. In the MALDI spectrum of GST P1-1 incubated with tyrosinase (Fig. 4b) a few additional peaks (a, b, c and d) appear from tyrosinase *per se*, but no change in the GST P1-1 ion pattern was observed. In contrast, an increase in the $2M^+$ signal, was observed, when GST P1-1 was incubated with eugenol in the presence of tyrosinase (Fig. 4c). With the strong reducing agent dithiotreitol (DTT) the mass spectrum of GST P1-1, inhibited with eugenol in the presence of tyrosinase, did not change, indicating that the inhibition is not reversible by reduction. The changes in the intensity distribution of the ion signals therefore suggest that a covalent binding is formed between the two subunits of GST P1-1 (Fig. 4c, $[2SU]^+$). However, due to the poor mass resolution of the dimer signal of GST P1-1, it was not possible to identify an accurate mass gain of 164.2 corresponding to the eugenol moiety. However, besides a clear broadening of the M^+ peak (Fig. 4c), the M^+ peak shifted slightly to higher values (Fig. 4c), which may indicate a mass gain of the subunit. After incubation of eugenol with GST A1-1 and M1a-1a in the presence of tyrosinase, no eugenol-related changes in the GST A1-1 and M1a-1a ion patterns were observed.

A



B

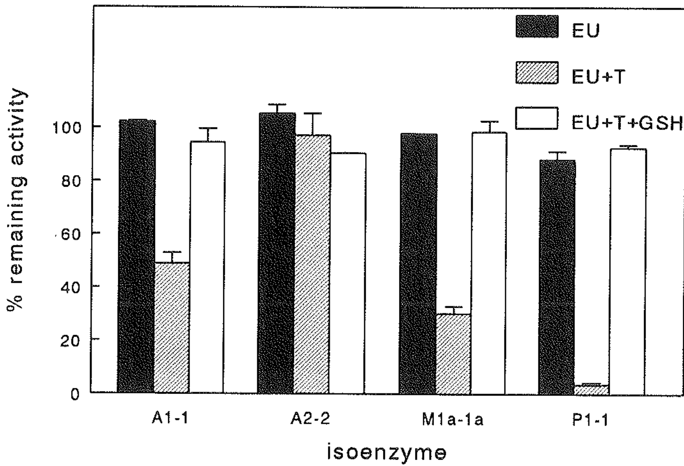


Fig. 3. Irreversible inhibition of purified (A) rat GST isoenzymes or (B) human GST isoenzymes by eugenol (EU) in the absence and presence of tyrosinase (T) and glutathione (GSH). 0.5 μM GST was incubated with 100 μM eugenol in the absence/presence of 100 units/ml tyrosinase (with and without further addition of 1 mM glutathione) for 30 min at room temperature. Blanc incubations were performed in the same way, using ethanol as solvent control. The catalytic activity was determined towards CDNB. Values are the average of two incubations and expressed as % remaining activity of blanc incubation ($\pm$ SD). For experimental details see Materials and methods.

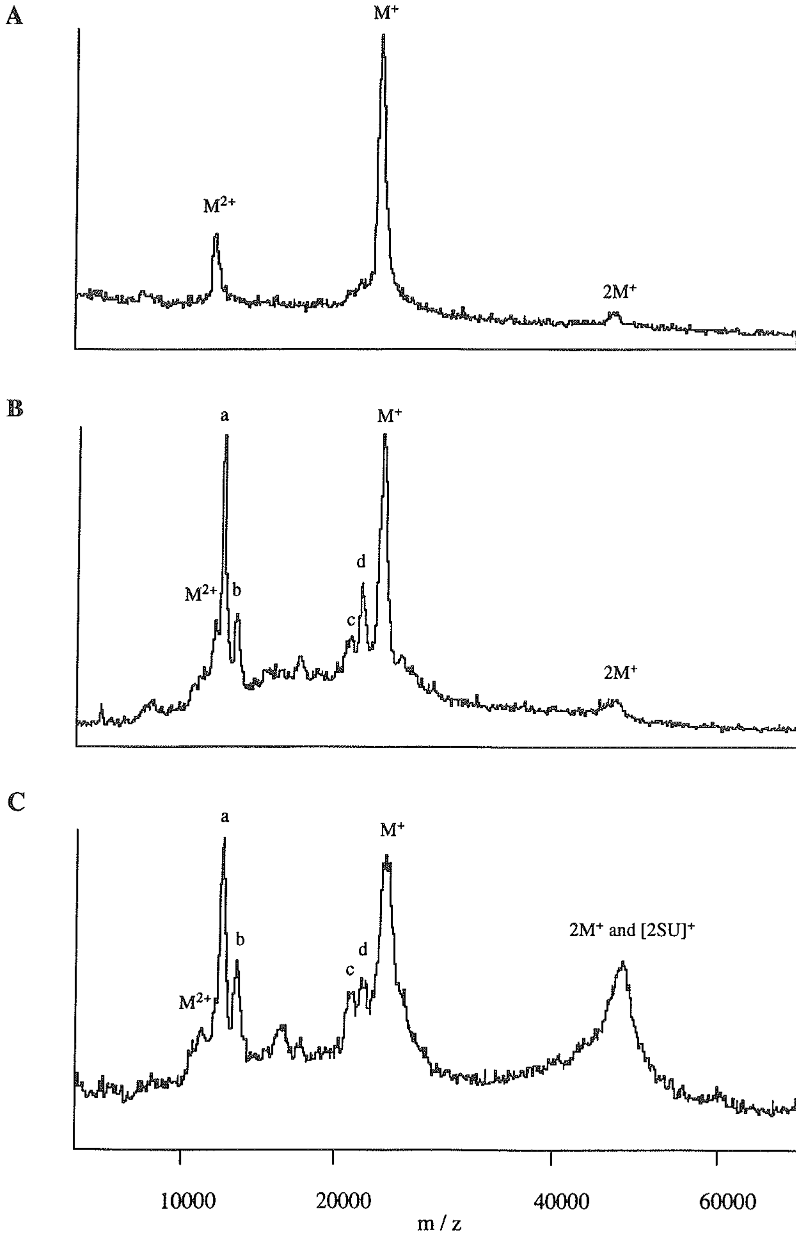


Fig. 4. Matrix-assisted laser desorption/ionization mass spectra of GST P1-1 (a) native, (b) incubated with tyrosinase, (c) incubated with eugenol and tyrosinase.

Table 1. I_{25} (mM)-values towards CDNB of rat GST incubated with eugenol (EU), eugenol methyl ether (EME), isoeugenol methyl ether (IME), 2-allylphenol (2-AP) or 4-propylphenol (4-PP).

Enzyme	EU	EME	IME	2-AP	4-PP
α-class					
GST 1-1	**4.5**	**0.6***	**1.6***	**0.3***	**4.6***
	(3.7-6.0)	(0.4-0.9)	(1.0-3.8)	(0.2-0.4)	(2.8-6.4)
GST 2-2	**>5.0**	**>2.5**	**>2.5**	**4.5**	**0.9**
				(3.9-5.1)	(-0.3-1.9)
μ-class					
GST 3-3	**0.7***	**0.5***	**0.4***	**4.4**	**1.7**
	(0.5-0.9)	(0.3-0.6)	(0.4-0.5)	(3.8-5.0)	(1.1-2.3)
GST 4-4	**2.1**	**1.3**	**>2.5**	**4.9**	**1.8**
	(1.8-2.5)	(1.0-1.7)		(4.4-5.4)	(1.0-2.5)
π-class					
GST 7-7	**0.8***	**1.2**	**1.7**	**3.6**	**1.6**
	(0.6-1.1)	(1.0-1.4)	(1.4-2.1)	(3.1-4.1)	(1.0-2.2)

20-30 nM enzyme was incubated with different concentrations of inhibitor, after which the enzymatic activity was determined towards CDNB. At least five concentrations were performed as described in Materials and methods.

[a] The concentration of inhibitor resulting in 25% inhibition of activity towards CDNB (I_{25}), calculated with the linear function $y = ax + b$ or the log linear function $y = a\ln(x) + b$.

Asterisk indicates I_{25}, calculated with the log linear function.

95% confidence interval is given in parentheses.

Reversible inhibition

All inhibitors were dissolved until saturation point. Due to the low saturation point of eugenol methyl ether and isoeugenol methyl ether the comparison of the five compounds by the I_{50} is hampered. Therefore, the I_{25} was calculated to compare the five compounds (Tables 1 and 2). For eugenol, the μ-class enzymes rat GST 3-3 (Table 1) and human GST M1a-1a (Tables 2) were the most sensitive enzymes. No drastic changes in sensitivity were seen by introducing other structural elements. In general, all compounds caused moderate reversible inhibition. In rat, the isoenzymes were most strongly inhibited by eugenol methyl ether. Thus, replacement of the hydroxyl group of eugenol by a methoxy-group leads to a stronger inhibition of GST in rat. In human, the overall inhibiting capacities of eugenol, eugenol methyl ether, isoeugenol methyl ether and 4-propyl phenol were more or less similar, whereas 2-allylphenol was the poorest inhibitor. Thus, the position of the allylic side chain seems also to play a role in the inhibition of human

Table 2. I_{25} (mM)-values towards CDNB of human GST incubated with eugenol (EU), eugenol methyl ether (EME), isoeugenol methyl ether (IME), 2-allylphenol (2-AP) or 4-propylphenol (4-PP).

Enzyme	EU	EME	IME	2-AP	4-PP
α-class					
A1-1	**0.5***	**1.8**	**>2.5**	**1.1**	**0.6**
	(0.3-0.7)	(1.5-2.3)		(-1.1-2.6)	(0.1-1.0)
A2-2	**4.1**	**>2.5**	**2.3**	**5.4**	**1.3**
	(3.7-4.6)		(1.9-3.2)	(4.9-5.8)	(0.2-2.2)
μ-class					
M1a-1a	**0.3***	**0.2***	**1.3**	**1.7**	**0.9**
	(0.3-0.4)	(0.1-0.3)	(1.2-1.5)	(1.4-1.9)	(0.7-1.0)
π-class					
P1-1	**>5.0**	**1.8**	**2.5**	**5.0**	**3.3**
		(1.6-2.0)	(2.1-3.2)	(4.4-5.6)	(3.2-3.5)

10-30 nM enzyme was incubated with different concentrations of inhibitor, after which the enzymatic activity was determined towards CDNB. At least five concentrations were performed as described in Materials and methods.
[a] The concentration of inhibitor resulting in 25% inhibition of activity towards CDNB (I_{25}), calculated with the linear function $y= ax + b$ or the log linear function $y= a\ln(x) + b$.
Asterisk indicates I_{25}, calculated with the log linear function.
95% confidence interval is given in parentheses.

GSTs: movement of the allylic side chain from the para-position to the ortho-position leads to less inhibition of GST isoenzymes.

Discussion

Recently, we found differences in GST activity *in vivo* between rat, mouse and man treated with eugenol. In liver of rats, oral administration of eugenol resulted in an induction of total GST activity [6,16,26], whereas in liver of mice on an eugenol-diet an inhibition of total GST activity was found [12]. In man, ingestion of eugenol resulted in decrease in α-class GSTs in plasma [18]. The latter findings *in vivo* can be explained by an irreversible GST inhibition by the oxidation product of eugenol, likely a quinone methide. Indeed, mouse and man are more sensitive to irreversible GST inhibition than rat. This may (partly) explain why *in vivo* no inhibition but rather an increase of GST activity is seen in eugenol-treated rats [6,16,26].

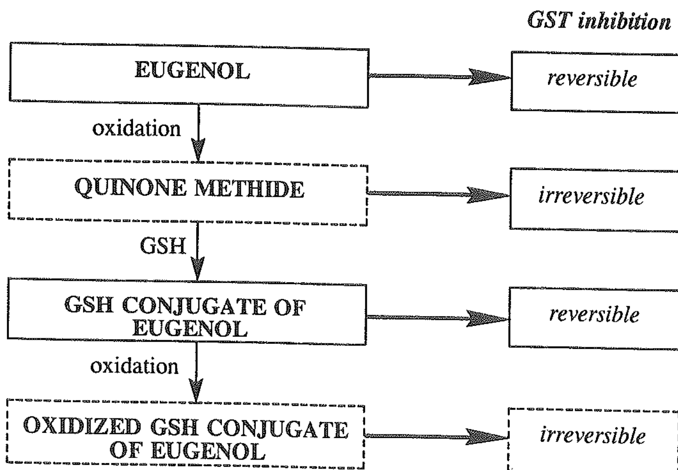


Fig. 5. Overview of inhibition of human GST by eugenol and its oxidation product (--- = hypothetical).

To study the effect of eugenol itself and its oxidation products on the GST classes α, μ and π, purified GST isoenzymes were used. Eugenol itself did not irreversibly inhibit the purified GST isoenzymes of rat and man but in the presence of tyrosinase eugenol irreversibly inhibited the human GST isoenzymes A1-1, M1a-1a and P1-1 and the rat GST isoenzymes 1-1, 2-2, 3-3, 4-4 and 7-7. In this respect the GSTs of the π-class (human GST P1-1 and rat GST 7-7) were by far the most sensitive enzymes. The human GST A2-2, which does not possess any cysteine residues [27], was not inactivated, suggesting that a cysteine residue is presumably the target site. Similar results were obtained with caffeic acid and dopamine [28,29]. It is well established that the GST of π-class possess a highly reactive cysteine residue, modification of which results in enzyme inactivation [30,31]. The cysteine residues of the GST π-class may undergo a (reversible) oxidative inactivation, by the formation of an intersubunit disulfide between the cysteines at the 47th and 101th-position [32,33,34].

Using mass spectrometry evidence was found that a covalent binding occurs between two subunits of GST P1-1 after incubation with eugenol in the presence of tyrosinase. These findings suggest that three mechanisms may be involved in the inhibition of GST P1-1: I) nucleophilic addition, II) oxidation of cysteine residues and III) covalent cross-linking of the subunits.

Further studies are needed to evaluate the toxicological implications of the present

findings. High levels of GST activity, particularly of the π-class, have been detected in rapidly growing tumor cells in rodents [35] and in humans [36]. GSTs of the α-class [35,37], μ-class [38] and especially π-class [39] may have an important role in tumor cell resistance to anti-cancer drugs. Current interest, therefore, is directed to the search for relatively non-toxic GST inhibitors to improve the effectiveness to cytostatic drugs. In this light, the glutathione conjugate of eugenol (resulting from the addition of glutathione to the quinone methide) [8,10], with its preferential inhibition of π-class GSTs in man, may be a very interesting compound (Fig. 5). Both the presence of a glutathione moiety with high affinity for GST and the oxidizible phenolic group make this compound a potential *in vivo* GST inhibitor. This is supported by the results obtained in humans *in vivo* [18]. Our results of the reversible human GST inhibition studies with structurally related compounds of eugenol indicate that the structural changes studied do not result in stronger reversible inhibition of GST in humans. In view of these findings, further studies into the action of the metabolite of eugenol responsible for the GST inhibition and its (potential) formation *in vivo* is warranted.

Acknowledgements

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CHAPTER 9

SUMMARIZING DISCUSSION AND CONCLUSIONS

It has been suggested that the use of antimutagens and anticarcinogens in everyday life will be the most effective procedure for preventing cancer and genetic disease (Ferguson, 1994). Therefore, much attention is being devoted to investigate compounds in foods with antimutagenic and/or anticarcinogenic potential. Currently, the naturally occurring minor non-nutrients are under investigation in the search for inhibitory compounds. The purpose of this thesis was to obtain insight into the antigenotoxic potential of one of these compounds, eugenol, the main component of clove oil.

(Geno)toxicity of eugenol

In studying antimutagenesis/anticarcinogenesis one has to be aware of possible adverse effects of inhibitors. The fact that a compound exerts an inhibitory effect against mutagenesis/carcinogenesis in an experimental system does not necessarily mean that it will be beneficial to the host. In case of dietary antigenotoxic/anticarcinogenic compounds, a beneficial effect is only valuable in the absence of (geno)toxicity.

Toxic effects of eugenol (weight loss and liver enlargement) have only been detected in studies using very high doses of eugenol ($\geq$1400 mg/kg or 1.2% in the diet) (Hagan *et al.*, 1965; NTP, 1983).

The genotoxicity of eugenol has been studied in many different studies both *in vitro* and *in vivo* (for an overview, see Chapter 1, part II). *In vitro*, eugenol shows little evidence for genotoxicity: eugenol was found to be genotoxic only in the chromosomal aberration test; in the unscheduled DNA synthesis assay, the Ames test and the sister chromatid exchange test eugenol was negative.

In vivo, most of the genotoxicity studies performed were negative, although there is limited evidence for genotoxicity of eugenol in the bone marrow micronucleus test and the ^{32}P-postlabelling assay. In the bone marrow micronucleus studies, i.p. administration of eugenol resulted in induction of micronucleated polychromatic erythrocytes (MPE) (Woolverton *et al.*, 1986; Ellahueñe *et al.*, 1994). Oral administration induces MPE only

in a very high dose (15 g/kg) (Woolverton *et al.*, 1986). In other bone marrow micronucleus studies with eugenol no effects on the incidence of MPEs have been found (Chapters 4 and 5; Maura *et al.*, 1986; Hayashi *et al.*, 1984; Ishidate *et al.*, 1988; Shelby *et al.*, 1993). Several studies on DNA-adduct formation of eugenol have reported that eugenol did not produce detectable amounts of adducts in liver DNA of eugenol-treated mice (Randerath *et al.*, 1984; Phillips *et al.*, 1984; Phillips, 1990). However, DNA-adduct formation in the liver was detected after prolonged exposure of mice to eugenol (0.4% in the diet) (Steenwinkel *et al.*, 1995; Chapter 6). This may be caused by DNA alkylation of a quinone methide of eugenol formed by oxidation of eugenol (Thompson *et al.*, 1992; Thompson *et al.*, 1995; Bolton *et al.*, 1995). The relevance of the DNA adduct for the genotoxicity of eugenol is questionable, since in the transgenic mouse mutation assay in the same study, described in Chapter 6, no indication for genotoxicity of eugenol was found. On the basis of these results, in combination with the carcinogenicity data (see Chapter 1, part II), it can be concluded that there is only limited evidence for genotoxicity and carcinogenicity for eugenol at very high dose levels.

Antigenotoxicity of eugenol

The results of the studies on the antigenotoxic potential of eugenol *in vitro* are summarized in Table 1. *In vitro*, a decrease of the mutagenicity induced by benzo[a]pyrene (B[a]P) was found in the Ames test using S9 or microsomes of eugenol-treated rats (Chapter 4; Yokota *et al.*, 1986). Direct addition of eugenol to the established mutagens aflatoxin B1 (AFB1), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and dimethylbenzanthracene (DMBA) resulted in a decrease of mutagenicity in the Ames test (Francis *et al.*, 1989; Amonkar *et al.*, 1986). Unfortunately, these decreases in genotoxicity of AFB1, DMBA and B[a]P induced by eugenol were not univocal: in other *in vitro* studies (e.g. the unscheduled DNA synthesis assay and the single-cell gel electrophoresis assay) described in Chapter 4 no effect on the genotoxicity induced by AFB1, DMBA and an increase of the genotoxicity induced by B[a]P was found. Thus, it can be concluded that eugenol has some antimutagenic potential *in vitro*, although the results are not univocal.

An overview of the effect of eugenol on the genotoxicity of established mutagens/carcinogens *in vivo* (in experimental animals and in human subjects) is given in Tables 2 and 3. The *in vivo* studies described in Chapters 5 and 6 show that eugenol has no effect on the genotoxicity induced by B[a]P in the bone marrow micronucleus test, in the transgenic mouse mutation assay and in the ^{32}P-postlabelling assay using an analysis method with

chromatographic conditions for B[a]P-DNA adducts. However, in the ^{32}P-postlabelling assay with an alkenylbenzene solvent system, the amount of B[a]P-DNA adducts was lower in mice fed the eugenol diet than in mice fed the control diet; the difference was not statistically significant but may yet be biologically relevant. This may indicate some antigenotoxic potential of eugenol *in vivo*. In the bone marrow micronucleus assay a clear antigenotoxic effect of eugenol was found: in eugenol-treated mice the genotoxicity induced by cyclophosphamide was significantly reduced as compared to control mice. It is concluded that in experimental animals, eugenol reveals its antigenotoxic potential *in vivo* to some extent. However, the study described in Chapter 7 shows that in human subjects consuming 150 mg eugenol for seven days there are no indications for an antigenotoxic potential of eugenol. The present data in human subjects are too few to conclude that no antigenotoxic potential *in vivo* is evident in man. Further studies are needed to study the relevance of antimutagenicity of eugenol to man.

Effect of eugenol on biotransformation enzymes

One important property of eugenol, which makes it a candidate antimutagen/anticarcinogen, is the induction of the phase-II biotransformation enzymes glutathione S-transferase (GST) and glucuronyl transferase (GT) (Yokota *et al.*, 1988; Zheng *et al.*, 1992). In these studies, this property was examined in more detail in rats, mice and human subjects (Table 4). In two subacute studies with rats described in Chapters 3 and 4, it was concluded that eugenol is no effective inducer of cytochrome P-450 enzyme activities, whereas an induction of phase-II biotransformation enzymes (GST and GT) was found indeed. In contrast, in Chapter 6 it is shown that in mice, dietary treatment with eugenol for 58 days resulted in a 50% decrease in GST activity and in no change in GT activity. In human subjects (Chapter 7), ingestion of 150 mg eugenol over 7 days resulted in a 26% decrease in α-class GSTs in plasma. The latter finding *in vivo* can be explained by an irreversible inhibition of GST by the oxidation product of eugenol, likely a quinone methide. Indeed, the *in vitro* studies described in Chapter 8 show that mouse and man are more sensitive to irreversible GST inhibition than the rats. This may (partly) explain why *in vivo* no inhibition but rather an increase in GST activity is seen in eugenol-treated rats (Yokota et al., 1988; Chapters 3 and 4).

In Chapter 8 it is also shown that eugenol in the presence of tyrosinase irreversibly inhibited human and rat GST isoenzymes of the α-, μ- and π-classes. In this respect the GSTs of the π-class were by far the most sensitive enzymes.

Based on the present results, it can be concluded that one of the properties that render eugenol a possible candidate antimutagen/anticarcinogen, namely induction of detoxifying phase-II biotransformation enzymes, is not found in man. In contrast, in the search for relatively non-toxic GST inhibitors to improve the effectiveness to cytostatic drugs in humans, the glutathione conjugate of eugenol (resulting from the addition of glutathione to the eugenol quinone methide) may be a very promising compound by virtue of its preferential inhibition of π-class GSTs.

Perspectives

In the studies described in this thesis one property of eugenol, i.e. induction of detoxifying phase II biotransformation enzymes, that renders it a possible candidate antimutagen/anticarcinogen was examined. Other properties, such as the antioxidant activity and the anti-nitrosating activity, still remain to be studied *in vivo*. Thus, although we did not find indications for an antimutagenic/anticarcinogenic activity of eugenol in man, eugenol still is a candidate antimutagen/anticarcinogen. Further studies are needed to determine whether eugenol may be beneficial to man. Such further studies should include: (1) identification of the metabolite responsible for the induction of GST and GT in rats; (2) determination of the concentrations of the metabolites that are responsible for induction and inhibition of GST at the relevant targets; (3) finding out whether there is a relation between inhibition and induction of GSTs.

In general, the field of antimutagenesis and anticarcinogenesis is still developing. At this time it is still difficult to comprehend the precise role of chemopreventive agents in foods in terms of reducing cancer incidence. Optimal chemopreventers should prevent carcinogenesis, or at least a particular cancer in an organ, without inducing toxicity or promoting carcinogenicity in other organs. To achieve this goal much more work is required. More chemopreventive compounds have to be identified and their effects have to be confirmed *in vivo*. Intervention studies examining chemopreventive compounds have to be performed to clarify the role of chemopreventive compounds in disease prevention and to identify compounds that may be applicated in designer foods. Until now the best suggestion for the consumer is: consume in moderation and include in your diet a variety of foods (in particular fruits and vegetables).

Conclusions

The data presented in the present thesis allow for the following conclusions:

1. It cannot be excluded that eugenol *per se* is genotoxic *in vivo* due to formation of a quinone methide.
2. On the basis of the limited evidence for genotoxicity and carcinogenicity, which becomes effective at very high dose levels, it is justified to study the potential beneficial effects of eugenol.
3. There is some evidence for an antigenotoxic potential of eugenol *in vitro*, but the results are not univocal.
4. There is some evidence for an antigenotoxic potential of eugenol *in vivo*, but the results are not univocal.
5. Consumption of 150 mg eugenol daily for seven days has no toxic effects in man.
6. There is no indication for an antigenotoxic potential of eugenol in man when 150 mg eugenol is consumed daily for seven days.
7. In rats, eugenol is an inducer of the phase-II biotransformation enzymes GST and GT.
8. Mouse and man are more sensitive to irreversible GST inhibition by oxidized eugenol than the rat.
9. GSTs of the π-class are by far the most sensitive enzymes for irreversible GST inhibition by eugenol in man.
10. Eugenol is a potential chemopreventive agent.

Table 1. Modulation of genotoxicity by eugenol *in vitro*.

End-point	Test system	Mutagen/carcinogen		Eugenol dose (treatment)		Result[a]	Reference
		Name	Concentration (treatment time)	*in vivo*	*in vitro*		
Primary DNA damage	unscheduled DNA synthesis assay (rat hepatocytes)[b]	DMBA	9.8 μM (18 h)	500 mg/kg (10x,po)	n.a.	-	Chapter 4 (Rompelberg *et al.*, 1995a)
				1000 mg/kg (10x,po)	n.a.	-	
		AFB_1	0.1 μM (18 h)	500 mg/kg (10x,po)	n.a.	-	
				1000 mg/kg (10x,po)	n.a.	-	
Gene mutation	*Salmonella typhimurium* (TA100)[c]	B[a]P	20 nmol/plate	500 mg/kg (10x,po)	(S9)	-	Chapter 4 (Rompelberg *et al.*, 1995a)
				1000 mg/kg (10x,po)	(S9)	↓	
			40 nmol/plate	500 mg/kg (10x,po)	(S9)	↑	
				1000 mg/kg (10x,po)	(S9)	↓	
			80 nmol/plate	500 mg/kg (10x,po)	(S9)	-	
				1000 mg/kg (10x,po)	(S9)	↓	
	Salmonella typhimurium (TA100)[c]	DMBA	40 nmol/plate	500 mg/kg (10x,po)	(S9)	-	Chapter 4 (Rompelberg *et al.*, 1995a)
				1000 mg/kg (10x,po)	(S9)	-	
			80 nmol/plate	500 mg/kg (10x,po)	(S9)	-	
				1000 mg/kg (10x,po)	(S9)	-	
			120 nmol/plate	500 mg/kg (10x,po)	(S9)	-	
				1000 mg/kg (10x,po)	(S9)	-	
	Salmonella typhimurium (TA 100)[d]	B[a]P	20 nmol/plate	800 mg/kg (4x,po)	(S9, micr.)	↓	Yokota *et al.*, 1986
			40 nmol/plate	800 mg/kg (4x,po)	(S9, micr.)	↓	
			80 nmol/plate	800 mg/kg (4x,po)	(S9, micr.)	↓	

	Salmonella typhimurium (TA 100)	AFB_1	1 nmol/ plate	n.a.	0.1 μmol/plate	-	Francis *et al.*, 1989
					0.5 μmol/plate	↓	
		MNNG	14 nmol/ plate	n.a.	0.1 μmol/plate	↓	
					0.5 μmol/plate	↓	
	Salmonella typhimurium (TA 98)	DMBA	10 nmol/plate	n.a.	0.1 μmol/plate	-	Amonkar *et al.*, 1986
					0.2 μmol/plate	↓	
					0.3 μmol/plate	↓	
					0.7 μmol/plate	↓	
					1.4 μmol/plate	↓	
Sister chromatid exchanges	CHO cells	MMC	150 nM (21 h)	n.a.	3.3 μM (pt,21 h)	-	Sasaki *et al.*, 1989
					10 μM (pt,21 h)	-	
					33.3 μM (pt,21 h)	-	
					100 μM (pt,21 h)	-	
Chromosomal aberration	CHO cells	AC	0.6 mM (3 h)	n.a.	30 μM (si,3 h)	-	Stich *et al.*, 1981
					61 μM (si,3 h)	-	
					122 μM (si,3 h)	-	
			1.3 mM (3 h)	n.a.	30 μM (si,3 h)	-	
					61 μM (si,3 h)	-	
					122 μM (si,3 h)	↑	
			2.6 mM (3 h)	n.a.	30 μM (si,3 h)	↑	
					61 μM (si,3 h)	↑	
					122 μM (si,3 h)	↑	

Table 1. *-continued-*

Comet	single-cell gel electrophoresis assay (Hep G2 cells)	B[a]P	15 μM (24 h)	n.a.	3 μM (pr,15 h)	-	Chapter 4 (Rompelberg *et al.*, 1995a)
			15 μM (24 h)		10 μM (pr,15 h)	-	
			15 μM (24 h)		30 μM (pr,15 h)	-	
			15 μM (24 h)		100 μM (pr,15 h)	-	
		B[a]P	15 μM (24 h)	n.a.	3 μM (ps,39 h)	-	
			15 μM (24 h)		10 μM (ps,39 h)	↑	
			15 μM (24 h)		30 μM (ps,39 h)	↑	
			15 μM (24 h)		100 μM (ps,39 h)	↑	
		B[a]P	15 μM (24 h)	n.a.	3 μM (si,24 h)	-	
			15 μM (24 h)		10 μM (si,24 h)	↑	
			15 μM (24 h)		30 μM (si,24 h)	↑	
			15 μM (24 h)		100 μM (si,24 h)	↑	
		B[a]P	15 μM (24 h)	n.a.	3 μM (pt,15 h)	-	
			15 μM (24 h)		10 μM (pt,15 h)	-	
			15 μM (24 h)		30 μM (pt,15 h)	-	
			15 μM (24 h)		100 μM (pt,15 h)	-	

[a] ↓ = decrease of genotoxicity of an established mutagen/carcinogen by treatment with eugenol; - = no effect of eugenol on the genotoxicity of an established mutagen/carcinogen; ↑ = increase of genotoxicity of an established mutagen/carcinogen by treatment with eugenol.
[b] hepatocytes of eugenol-treated Wistar rats (♂) have been used.
[c] S9 of eugenol-treated Wistar rats (♂) has been used.
[d] S9 and microsomes of eugenol-treated Wistar rats (♂) have been used.
AC=arecoline; AFB1= aflatoxin B1; B[a]P= benzo[a]pyrene; DMBA= dimethylbenzanthracene; MMC= mitomycin C; MNNG= N-methyl-N'-nitro-N-nitrosoguanidine; n.a.= not applicable; po= *per os*; pr= pre-treatment; ps= pre- and simultaneous treatment; pt= post-treatment; si= simultaneous treatment.

Table 2. Modulation of genotoxicity by eugenol *in vivo* (experimental animals).

End-point	Test system	Species	Mutagen/carcinogen (dose;treatment)	Eugenol dose (treatment)	Killing time[a]	Result[b]	Reference
Micronuclei (bone marrow)	Bone marrow mutagenicity assay	Swiss mice (♂)	CP (25 mg/kg;1x,ip)	0.4% in diet (15 days)	24 h	↓	Chapter 5 (Rompelberg *et al.*, 1995b)
			mitomycin C (1.5 mg/kg;1x,ip)	0.4% in diet (15 days)	24 h	-	
			EMS (300 mg/kg;1x,ip)	0.4% in diet (15 days)	24 h	-	
			B[a]P (250 mg/kg;1x,ip)	0.4% in diet (15 days)	24 h	-	
DNA adducts (liver)	^{32}P-postlabelling assay for B[a]P-DNA adducts	λ-*Lac*Z-transgenic mouse strain 40.6 (Muta^{TM}Mouse) (♂)	B[a]P (100 mg/kg; 1x,ip)	0.4% in diet (58 days)	48 days	-	Chapter 6 (Rompelberg *et al.*, 1995c)
	^{32}P-postlabelling assay for alkenylbenzene-DNA adducts	λ-*Lac*Z-transgenic mouse strain 40.6 (Muta^{TM}Mouse) (♂)	B[a]P (100 mg/kg; 1x,ip)	0.4% in diet (58 days)	48 days	-	Chapter 6 (Rompelberg *et al.*, 1995c)
*Lac*Z mutants (liver)	transgenic mouse mutation assay	λ-*Lac*Z-transgenic mouse strain 40.6 (Muta^{TM}Mouse) (♂)	B[a]P (100 mg/kg;1x,ip)	0.4% in diet (58 days)	48 days	↓	Chapter 6 (Rompelberg *et al.*, 1995c)

[a] Time after treatment with an established mutagen/carcinogen.
[b] ↓ = decrease of genotoxicity of an established mutagen/carcinogen by treatment with eugenol.
- = no effect of eugenol on the genotoxicity of an established mutagen/carcinogen.
B[a]P= benzo[a]pyrene; CP= cyclophosphamide; EMS= ethyl methanesulphonate; ip= intraperitoneal.

Table 3. Effect of short-term dietary administration of eugenol in humans (♂) on the genotoxicity of established mutagens.

End-point	Test system	Mutagen[a] (dose;treatment time)	Eugenol dose (treatment time)	Result[b]	Reference
Micronuclei (lymphocytes)	micronucleus assay	vinblastine (55 nM; 2 h)	150 mg (7 days)	-	Chapter 7 (Rompelberg *et al.*, 1995d)
		mitomycin C (0.9 and 1.5 μM; 2 h)	150 mg (7 days)	-	
Chromosome aberrations (lymphocytes)	chromosomal aberration assay	mitomycin C (0.9 and 1.5 μM; 2 h)	150 mg (7 days)	-	Chapter 7 (Rompelberg *et al.*, 1995d)

[a]blood samples of men consuming eugenol for 7 days were treated *in vitro* with an established mutagen.
[b]- = no effect of consumption of eugenol for 7 days on the genotoxicity of an established mutagen *in vitro*.

Table 4. Modulation of phase I and II biotransformation enzymes by eugenol in liver of rat and mouse and in human blood samples.

Species	Eugenol dose (treatment time and route)	Cytochrome P450 activities total	1A	1A1	2B	2B1	2C11	3A	GST[a]	GSTα	GT+ ST[b]	GT[c]	GT[d]	DTD	Reference
Wistar rat (♂)	250 mg/kg (10x, po)	-	-		-				-			↑	-	-	Chapter 3 (Rompelberg *et al.*, 1993)
	500 mg/kg (10x, po)	-	-		-				↑			↑	↑	-	
	1000 mg/kg (10x, po)	-	↑		↑				↑			↑	↑	-	
Wistar rat (♂)	500 mg/kg (10x, po)	-		-		-	-	-	↑						Chapter 4 (Rompelberg *et al.*, 1995a)
	1000 mg/kg (10x, po)	-		-		↑	-	-	↑						
λ-*LacZ*-transgenic mouse strain 40.6 (Muta™Mouse) (♂)	0.4% in diet (58 days)								↓			-	-		Chapter 6 (Rompelberg *et al.*, 1995c)
Human (♂)	150 mg (7 days, po)								-	↓	-				Chapter 7 (Rompelberg *et al.*, 1995d)

[a] determined in liver cytosol of experimental animals and in human erythrocytes; [b] determined with paracetamol-test (see Chapter 7); [c] determined with 4-chlorophenol as substrate; [d] determined with 4-hydroxybiphenyl as substrate.
↓ = decrease of biotransformation enzyme activity by treatment with eugenol; - = no effect of eugenol on biotransformation enzyme activity;
↑ = increase of biotransformation enzyme activity by treatment with eugenol.
DTD= DT-diaphorase; GST= glutathione S-transferase; GSTα= α-class GST; GT= glucuronyl transferase; ST= sulphotransferase; po= *per os*.

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Samenvatting voor niet-vakgenoten

In onze voeding komen zowel schadelijke als heilzame stoffen voor. Ongeveer 30% van de kankergevallen met dodelijke afloop wordt toegeschreven aan de voeding. Daarnaast bevat onze voeding stoffen die beschermen tegen kanker, de zogenaamde chemopreventieve stoffen. De laatste jaren is er veel belangstelling voor deze stoffen. Uit epidemiologische studiës is gebleken dat er een negatief verband bestaat tussen kanker en de consumptie van vezelrijke produkten, fruit en groenten.

Recent wordt onderzoek verricht naar de chemopreventieve werking van niet-nutriënten (stoffen zonder voedingswaarde) zoals kruiden. In dit proefschrift wordt de potentieel chemopreventieve werking onderzocht van eugenol, een stof die voornamelijk in kruidnagel voorkomt. Hierbij staat het effect van eugenol op de genotoxiciteit (beschadiging van het DNA)/mutageniteit (verandering in het DNA) en de biotransformatie (omzetting van lichaamsvreemde stoffen door het lichaam) centraal.

De biotransformatie is te verdelen in twee fasen: fase-I en fase-II. In fase-I worden lichaamsvreemde stoffen door enzymen omgezet in metabolieten, die reactief en schadelijk kunnen zijn. Vervolgens worden deze metabolieten in fase-II door conjugatie-enzymen zo veranderd dat het gevormde produkt het lichaam gemakkelijk kan verlaten.

De genotoxiciteit van een stof wordt onderzocht door middel van een stapsgewijze benadering. Dit houdt in dat de genotoxiciteit eerst *in vitro* wordt bestudeerd met prokaryotische of eukaryotische celsystemen en vervolgens *in vivo* bij proefdieren in kort- en langdurende studies. Deze gegevens kunnen aangevuld worden met humane gegevens uit biomerker-studies of epidemiologische studies. Dezelfde benadering kan worden gebruikt voor het onderzoeken van de modulatie van genotoxiciteit. Daartoe wordt de potentieel modulerende stof te samen met een genotoxische stof *in vitro* of *in vivo* getest. Indien de genotoxiciteit vermindert, noemt men de stof antigenotoxisch.

Ter inleiding wordt in hoofdstuk 1 een overzicht gegeven van chemopreventieve stoffen en hun werkingsmechanismen gevolgd door een toxicologisch profiel van eugenol.

Om een indicatie te krijgen van het gebruik van kruiden door de algemene bevolking, is in hoofdstuk 2 het gebruik van kruidnagel, peper, kerrie, nootmuskaat en knoflook onderzocht in twee epidemiologische studies.

In hoofdstuk 3 is het effect van eugenol op de biotransformatie onderzocht. In ratten was na orale toediening van eugenol een toename in de activiteit van de fase-II enzymen glutathion-S transferase (GST) en glucuronyl transferase (GT), te zien. Tevens werd een relatief kleine toename in de activiteit van fase-I enzymen, cytochroom P450-enzymen, waargenomen. Een toename in de activiteit van met name fase-II enzymen, duidt in het algemeen op een snellere uitscheiding van kankerverwekkende en andere schadelijke stoffen en wordt daarom gezien als een beschermend mechanisme tegen het ontstaan van

kanker. Om dit te verifiëren zijn verdere *in vitro* en *in vivo* testen uitgevoerd.

In hoofdstuk 4 is het effect van eugenol op de genotoxiciteit van drie bekende kankerverwekkende stoffen, benzo[a]pyreen (B[a]P), aflatoxine B1 (AFB1) en dimethylbenzanthraceen (DMBA), onderzocht in verschillende testen: de DNA-herstel-test, de Ames-test en de 'komeet-test' (enkele-cel-gel-electroforese-test). Uit ratten die eugenol toegediend kregen werd de lever geïsoleerd. De lever werd vervolgens *in vitro* in de DNA-herstel-test en de Ames-test blootgesteld aan B[a]P, DMBA en AFB1. In de komeet-test is het effect van *in vitro* behandeling met eugenol op de genotoxiciteit geïnduceerd door B[a]P onderzocht in een humane levercellijn.

De resultaten van de drie *in vitro* testen waren uiteenlopend: in de Ames-test was een afname te zien van de genotoxiciteit veroorzaakt door B[a]P, terwijl in de komeet-test juist een toename van de genotoxiciteit van B[a]P werd waargenomen. Eugenol had geen effect op de genotoxiciteit van AFB1 en DMBA. Dit betekent dat er slechts beperkte aanwijzingen zijn voor een antigenotoxische werking van eugenol.

In hoofdstuk 5 en 6 is het effect van eugenol op de genotoxiciteit geïnduceerd door B[a]P *in vivo* onderzocht in een beenmerg-micronucleus-test met muizen (hoofdstuk 5) en een λ-*lac*Z-mutatie-test met transgene muizen (hoofdstuk 6). In hoofdstuk 5 werden naast B[a]P ook cyclofosfamide (CP), mitomycine C en ethylmethaansulfonaat (EMS) gebruikt als genotoxische stof. Orale toediening van eugenol resulteerde in een significante afname van de genotoxiciteit veroorzaakt door CP, maar had geen effect op de genotoxiciteit veroorzaakt door mitomycine C en EMS. In de λ-*lac*Z-mutatie-test resulteerde orale toediening van eugenol in een (biologisch relevante) afname van B[a]P-DNA adducten in de levers van muizen die het eugenol-voer kregen. Orale toediening van eugenol had echter geen effect op de mutageniteit van B[a]P. Tevens werd een eugenol-geassocieerd DNA-adduct gevonden. Dit DNA-adduct kan veroorzaakt worden door binding van een metaboliet van eugenol, eugenol-chinonmethide, aan het DNA. De relevantie van dit DNA-adduct is echter twijfelachtig omdat in dezelfde dieren geen indicatie voor mutageniteit van eugenol werd gevonden in de λ-*lac*Z-mutatie-test.

Uit de resultaten van hoofdstuk 5 en 6 kan geconcludeerd worden dat er zowel indicaties zijn voor een antigenotoxisch effect van eugenol *in vivo* als voor een genotoxisch effect van eugenol zelf.

In hoofdstuk 6 is ook het effect van eugenol op de fase-II enzymen GST en GT in de muis onderzocht. In tegenstelling tot de rat, werd bij de muis een remming van de GST-activiteit en geen effect op de GT-activiteit gevonden.

In hoofdstuk 7 is de modulerende werking van eugenol op de genotoxiciteit en de biotransformatie door eugenol getest bij mensen. Tien gezonde vrijwilligers slikten dagelijks 150 mg eugenol of een placebo gedurende een week. Na een 'uitwas'-periode van een week werden de groepen die eugenol of de placebo slikten gewisseld en kregen beide groepen de andere behandeling gedurende een week. Om de antigenotoxiciteit van eugenol te testen werden op dag 8 en 22 bloedmonsters verzameld en *in vitro* blootgesteld

aan de genotoxische stoffen mitomycine C en vinblastine. Na blootstelling werd het percentage witte bloedcellen met chromosomale afwijkingen en micronuclei bepaald. Op dag 8 en 22 werd de fase-II biotransformatiecapaciteit (o.a. GT) bepaald. GST-activiteiten werden gemeten in het bloed. Er werd geen antigenotoxisch effect van eugenol gevonden. Wel werd een modulerend effect van eugenol op het fase-II biotransformatie-enzym GST gevonden bij mensen die eugenol geslikt hadden: α-klasse GST in het plasma was significant afgenomen na eugenolconsumptie in vergelijking met consumptie van de placebo. Dit kan betekenen dat GST geremd wordt door eugenol (evenals bij de muis) en/of dat eugenol beschermt tegen achtergrondschade van levercellen door eugenol.

In hoofdstuk 8 zijn de verschillen in het effect van eugenol op GST-enzymen van de rat, muis en mens nader onderzocht. *In vitro* werd aangetoond dat eugenol na oxidatie in staat is om GST irreversibel te remmen. GST-enzymen van achtereenvolgens rat, mens en muis worden in toenemende mate geremd. Dit kan de uiteenlopende *in vivo* effecten van hoofdstuk 3, 6 en 7 (gedeeltelijk) verklaren.

Op basis van de resultaten van dit proefschrift wordt in hoofdstuk 9 het onderstaande geconcludeerd:

1. Het kan niet uitgesloten worden dat eugenol *in vivo* genotoxisch is door de vorming van een chinonmethide.
2. Op basis van de beperkte aanwijzingen voor genotoxiciteit en carcinogeniteit, die optreden bij zeer hoge doseringen, is onderzoek naar het potentieel chemopreventieve effect van eugenol gerechtvaardigd.
3. Er zijn aanwijzingen voor een chemopreventief effect van eugenol *in vitro* maar de resultaten zijn niet eenduidig.
4. Er zijn aanwijzingen voor een chemopreventief effect van eugenol *in vivo* maar de resultaten zijn niet eenduidig.
5. Het dagelijks consumeren van 150 mg eugenol gedurende 7 dagen heeft geen toxisch effect op de mens.
6. Bij mensen die gedurende 7 dagen dagelijks 150 mg eugenol consumeren is geen antigenotoxisch effect van eugenol aangetoond.
7. Bij de rat induceert eugenol de fase-II enzymen GST en GT.
8. De mens en de muis zijn gevoeliger voor irreversibele GST-remming door geoxideerd eugenol dan de rat.
9. Bij de mens zijn GSTs van de π-klasse het gevoeligst voor irreversibele GST-remming door eugenol.
10. Eugenol is een potentieel chemopreventieve stof.

Curriculum Vitae

Cathy Rompelberg werd op 20 april 1967 geboren te Maastricht. In 1985 behaalde zij het gymnasium-ß diploma aan het Jeanne d'Arc college te Maastricht. In datzelfde jaar begon zij aan de studie Gezondheidswetenschappen, met als afstudeerrichting Biologische Gezondheidkunde, aan de Rijksuniversiteit Limburg te Maastricht. Tijdens de afstudeerstage heeft zij onderzoek gedaan op het gebied van de klinische voeding bij de afdeling Interne Geneeskunde van het Academisch Ziekenhuis Maastricht. Vervolgens heeft ze in 1990 onderzoek verricht bij het Laboratorium voor Carcinogenese en Mutagenese van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven op het gebied van de genetische toxicologie. Na een korte stage bij de afdeling Genetische Toxicologie van TNO-Voeding te Zeist, is ze in mei 1991 begonnen als assistent in opleiding bij het Utrechts Toxicologisch Centrum (UTOX). Het promotieonderzoek werd uitgevoerd bij TNO-Voeding. Naast het promotieonderzoek is de postdoctorale opleiding Toxicologie gevolgd. Sinds mei 1995 is zij als wetenschappelijk medewerkster aangesteld bij het Laboratorium voor Geneesmiddelen en Medische Hulpmiddelen van het RIVM te Bilthoven. In deze functie is zij betrokken bij de beoordeling van de preklinische toxicologie en farmacokinetiek van geneesmiddelen die ter registratie worden aangeboden bij het College ter Beoordeling van Geneesmiddelen.

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STELLINGEN

1. Eugenol: antimutageen in de knop.

2. Onderzoek naar het effect van een antimutagene/anticarcinogene stof *in vivo* bij de mens vereist een zorgvuldige afstemming van *in vitro*-, proefdier- en humaan onderzoek.

3. Als toediening van een chemische stof enerzijds tot vorming van een DNA-adduct leidt en anderzijds geen mutaties veroorzaakt, dient men de relevantie van het adduct voor de potentieel carcinogene werking van de stof in twijfel te trekken.
(*dit proefschrift*)

4. De mate van irreversibele GST-remming door geoxideerd eugenol *in vitro* is species-afhankelijk.
(*dit proefschrift*)

5. Voor de beoordeling van de toepasbaarheid van een chemopreventieve stof bij de mens is kennis van de toxiciteit vereist.
(*dit proefschrift*)

6. Het is een misvatting dat natuurlijk per definitie gezond is.

7. Het gebruik van proefdieren als een testsysteem voor het beoordelen van het risico dat mensen lopen die blootgesteld worden aan een chemische stof, is een opmerkelijk ambitieuze onderneming.
(*Monro and Mordenti, 1995, Toxicol. Pathol. 23, 187-198*)

8. Without publication, science is dead.
(*Gerald Piel*)

9. Uit de voorkeur van wetenschappelijke tijdschriften voor het publiceren van positieve studies boven negatieve studies blijkt dat de stelling 'geen resultaten zijn ook resultaten' voor publicaties vaak niet opgaat.

10. Regelmatig vakantie houden heeft een motiverende uitwerking op het promotieonderzoek.

Cathy Rompelberg

10 november 1995