Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in *Salmonella typhimurium* and detection of associated DNA adducts using UPLC–MS/MS methods

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Methyleugenol, a secondary metabolite present in many herbal spices, is carcinogenic in various tissues of mice and rats but negative in standard *in vitro* mutagenicity tests. Several observations indicate that hydroxylation followed by sulfation is an important activation pathway in the carcinogenicity and DNA adduct formation by methyleugenol and other alkenylbenzenes in animal models. However, sulfation is not taken into account in standard *in vitro* tests. Therefore, we have studied whether expression of murine or human sulfotransferases (SULTs) in the target strain, *Salmonella typhimurium* TA100, leads to the activation of hydroxylated metabolites of methyleugenol (\(\pm\)1′-hydroxymethyleugenol, (−)-1′-hydroxymethyleugenol and \((E)\)-3′-hydroxymethylisoeugenol). Human SULT1A1 (a form expressed at high levels in many tissues) and SULT1C2 (expressed primarily in foetal tissues) activated all three compounds under very low substrate concentrations. At higher concentrations, activation was also observed with human SULT1A2 and SULT1E1. Murine Sult1a1 required higher substrate concentrations than its human orthologue. Other SULT forms (human 1A3, 1C1, 1C3, 2A1 and 2B1b as well as murine 1d1) did not activate any methyleugenol metabolites studied. Furthermore, we developed isotope-dilution mass-spectrometric methods for the sensitive and specific detection of DNA adducts formed by methyleugenol metabolites. All three hydroxylated metabolites formed the same DNA adducts in *S. typhimurium* TA100-hSULT1A1: high levels of N\(^\circ\)-(trans-methylisoegenol-3′-yl)-2′-deoxyguanosine and modest levels of N\(^\circ\)-(trans-methylisoegenol-3′-yl)-2′-deoxyadenosine. Adduct levels correlated with the mutagenic effects induced. No adducts were formed by the test compounds in the SULT-deficient standard strain TA100. In conclusion, several methyleugenol metabolites are activated to DNA-reactive mutagens in *S. typhimurium* upon incorporation of appropriate sulfation capacity. We have identified human and murine SULT forms able to catalyse this activation. Methods were developed that may be utilised to analyse DNA samples from human tissues specifically for the possible presence of methyleugenol adducts.

Introduction

Methyleugenol is a secondary metabolite occurring in herbal spices, such as basil, pimento, laurel, anise and tarragon. Moreover, essential oils rich in methyleugenol are used for flavouring foods and cosmetics. The usage of pure methyleugenol for this purpose has been banned in some countries (e.g. the European Union) but not in others (e.g. USA). Methyleugenol induced tumours in various tissues of rats and mice (1,2). In particular, it is a potent hepatocarcinogen in both species. Methyleugenol and various other alkenylbenzenes form DNA adducts in liver and other tissues of rodent models, suggesting that they are genotoxic carcinogens (3–7). However, they are negative in the Ames test and other standard *in vitro* genotoxicity assays (2). In general, DNA adducts detected in animal tissues involved the binding of the 3′-C atom of the alkenylbenzenes to the exocyclic amino groups of purine bases (3–7). 1′-Hydroxylated metabolites administered to animals form the same DNA adducts, usually at higher levels than the parent alkenylbenzenes, and are carcinogenic, suggesting that they represent proximate genotoxicants/carcinogens. The sulfotransferase (SULT) family 1 inhibitor pentachlorophenol drastically reduced carcinogenicity and DNA adduct formation by safrole and 1′-hydroxysafrole in rat and mouse models (6–9). Likewise, tumorigenesis and DNA adduct formation by safrole was strongly reduced in brachymorphic mice, which are characterised by a reduced synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the cofactor for SULTs (7). These various findings suggested that the metabolic pathways exemplified for methyleugenol in Figure 1 are important for biological effects of alkenylbenzenes *in vivo*. It is important to notice that standard *in vitro* mutagenicity tests are deficient in sulfo conjugation activity due to the absence of SULT enzymes in the target cells and the lack of cofactor in the usual activating system, liver S9 mix (10,11). Supplementation of the cofactor PAPS to the S9 mix is not a reliable remedy, as sulfo conjugates are charged molecules, which do not efficiently penetrate cell membranes (10,12).

We have expressed various human and rodent SULTs in Ames’s *Salmonella typhimurium* strains (12,13). The objective of the present study was to find out whether hydroxylated metabolites of methyleugenol are activated to mutagens in this model. Whereas the role of sulfation is convincingly documented for genotoxic and carcinogenic activities of alkenylbenzenes in rodent models, the SULT forms involved have not yet been identified and it is not known whether human SULTs have similar activities. Therefore, recombinant bacteria were used in the present study to identify critical SULT forms. It has to be noted that 1′-hydroxymethyleugenol is a chiral molecule, existing as two enantiomers. Moreover, in addition to 1′-hydroxymethyleugenol, its isomer 3′-hydroxymethylisoegenol (existing as E and Z isomers) may be activated by SULT (Figure 1). We have not found any information on the enantiomeric composition of metabolically formed 1′-hydroxymethyleugenol.
However, we previously reported that hepatic microsomes from control and Aroclor 1254-treated rats form the (+)-enantiomer of 1'-hydroxysafrole from safrole with an enantiomeric excess of 0.45 and 0.60, respectively (14). The E diasteromer of 3'-hydroxymethylisoeugenol is thermodynamically favoured compared to the Z form. Only the E diasteromer but not the Z isomer is formed in incubations of methyleugenol with microsomes from rat, human and bovine liver (A. T. Cartus, K. Herrmann, L. W. Weishaupt, K. H. Merz, W. Engst, H. R. Glatt, D. Schrenk, manuscript in preparation). This raises the question whether the same or different SULT forms are involved in the activation of these isomeric phase-I metabolites of methyleugenol. We have investigated both enantiomers of 1'-hydroxymethyleugenol, but only the E diasteromer of 3'-hydroxymethylisoeugenol, as its Z does not appear to be formed metabolically. Finally, we devised mass spectrometric methods for identifying and quantifying DNA adducts in the test system.

Fig. 1. Bioactivation pathways of methyleugenol leading to electrophilic sulfuric acid esters. Chiral centres are marked with an asterisk.

### Materials and methods

**Chemicals**

$[^{15}N]_2$-Deoxyadenosine ($[^{15}N]$-dA) and $[^{15}N]_2$-deoxyguanosine ($[^{15}N]$-dG) were obtained from Silantes (München, Germany). Solvents (HPLC-grade) used for gas chromatography, high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Herring sperm DNA, micrococcal nuclease (from *Staphylococcus aureus*) and alkaline phosphatase (from calf intestine) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Calf spleen phosphodiesterase type II was obtained from Merck (Darmstadt, Germany).

**Nuclear magnetic resonance and gas chromatography analyses**

$^1$H-nuclear magnetic resonance (NMR) analyses of (+)-1'-hydroxymethyleugenol, $N^6$-(trans-methylisoeugenol-3'-yl)-2'-deoxyadenosine ($N^6$-MIE-dA) and $N^2$-(trans-methylisoeugenol-3'-yl)-2'-deoxyguanosine ($N^2$-MIE-dG) dissolved in dimethyl sulfoxide (DMSO)-d$_6$ were performed with an NMR500-mmns500 spectrometer (Varian, Palo Alto, California) at 298 K. (E)-3'-Hydroxymethyleugenol dissolved in acetonitrile-d$_3$ was investigated with a 500 MHz Inova spectrometer (Varian) at 298 K. Gas chromatography–mass spectrometry operating in electron impact mode (GC-EI-MS) analyses were performed with a CP3800 gas chromatograph (Varian) equipped with a J&W DB-5MS Column (0.25 × 30 mm, 0.25 μm film; Agilent Technology, Waldbronn, Germany) and a Varian 320-MS detector (Varian) recording m/z of 100–400. The injection volume was 1 μl. The flow rate of the carrier gas, helium, was 1.2 ml/min. Gas temperature was increased from 50°C (hold for 1 min) to 250°C (hold for 4 min) at a rate of 5°C/min. All other parameters were taken from Jeurissen et al. (15), as used for the analysis of (+)-1'-hydroxyestragole.

**Synthesis of hydroxylated metabolites of methyleugenol and (+)-1'‘-acetoxymethyleugenol**

(+)-1'-Hydroxymethyleugenol was prepared from 3,4-dimethoxybenzaldehyde (Sigma–Aldrich) using the same principal methods as described by Borchert et al. (16) for the synthesis of (+)-1'-hydroxyestragole. The crude product (yield up to 93%) had a purity of at least 98%. Samples were purified by chromatography to give racemic material of ≥99% purity for mutagenicity testing and $^1$H-NMR analyses. The $^1$H-NMR spectrum agreed with that described by Cartus et al. (17). Other samples of the crude product were separated into the enantiomers by HPLC (Dionex, Idstein, Germany) equipped with two sequentially arranged columns (each 4.6 × 250 mm, 10 μm) containing Chiralcel OJ00CE-BG054 and OJ00CE-GI037 (Daicel Industries, Tokyo, Japan), respectively, and a UV-970 Intelligent ultraviolet (UV)/visible light detector (Jasco, Groß-Umstadt, Germany) recording absorption at 278 nm. Aliquots (100 μl) of the crude material, containing ~5 mM 1'-hydroxymethyleugenol in n-heptane:ethanol (9:1), were injected. The same solvent mixture was used as isotopic eluent at a flow rate of 0.7 ml/min. One enantiomer (later identified as the (+) enantiomer) eluted at ~50 min and the
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other at ~55 min. After evaporation of the solvent, the purity of each enantiomer was >99% [no contamination with the other enantiomer or any other compounds detectable by HPLC-UV]. The polarity of the enantiomers was determined by circular dichroism spectroscopy using a Jasco-710 spectrometer (Jasco) at room temperature with a response time of 4 sec, a scanning speed of 50 nm/min and a wavelength range of 178–320 nm. Each enantiomer was dried in n-hexane to a final concentration of 0.5 mM and added to a quartz cell of 1-mm optical path length.

The enantiomers [2 μg/μl in n-heptane:ethanol (9:1)] were further analysed by GC-EL-MS with the following result: (+)-1′-hydroxymethyleugenol: m/z (relative intensity, %): 139 (100), 151 (47), 165 (63), 176 (55), 194 (96); (−)-1′-hydroxymethyleugenol: m/z (relative intensity, %): 139 (100), 151 (47), 165 (96), 176 (71), 194 (93). These fragmentations correspond to those reported for (+)-1′-hydroxyestrageola (18). The high purity of the enantiomers (>99%) previously determined by HPLC-UV was confirmed by this GC-EL-MS analysis.

(E)-3′-Hydroxymethyleugenol was prepared by isomerisation of (+)-1′-hydroxymethyleugenol using the same principal approach as described for the synthesis of (E)-3′-hydroxysafasfola (16). The yield before purification was up to 46%. Purification and 1H-NMR analysis were kindly performed by MicroCombiChem (Wiesbaden, Germany). Briefly, the crude product was dissolved in acetonitrile resulting in a concentration of nearly 50 mM (E)-3′-hydroxymethyleugenol. Aliquots of 250 μl were added into an HPLC equipped with a 2700 Sample Manager, an Atlantis dC18 column (19 × 100 mm, 5 μm) at a flow rate of 15 ml/min. Solvent B was increased over a period of 30 min from 20 to 50% for elution of dA adducts and from 10 to 40% for elution of dG adducts. Fractions containing the products were combined and subjected to HPLC purification. The DNA was recovered by precipitation with 16.5 ml ice-cold 2-MIE-dG in 15 N5 of 75% ethanol. Strahle (Germany) recording absorptions at 210 and 254 nm. A 7-min linear gradient was applied from 10% acetonitrile/0.1% formic acid (v/v) to 100% acetonitrile/0.1% formic acid (v/v). The flow was set at 18 ml/min. (E)-3′-Hydroxymethyleugenol eluted at 3.5 min. The 1H-NMR spectrum agreed with that reported by Cartus et al. (17). The purity was >99% according to this 1H-NMR analysis.

(+)-1′-Acetoxymethyleugenol was synthesised from (+)-1′-hydroxymethyleugenol using a protocol described for the acetylation of (+)-1′-hydroxyestrageola (19). The yield was 80%. A sample of the crude product [2 μg/μl in n-heptane:ethanol (9:1)] was analysed by GC-EL-MS with the following result for the main product: m/z (relative intensity, %): 105 (100), 161 (33), 176 (77), 177 (16), 194 (43), 236 (14). These fragmentations corresponded to those reported for the photodiode array detector (99) to 263 nm for dG adducts or 273 nm for dA adducts (all equipment from Waters). A linear gradient of water acidified with 0.25% acetic acid (v/v) and 0.25% formic acid (v/v) (solvent A) and acetonitrile (solvent B) was used for elution at a 5-fold higher density in medium A. Mutagenicity was determined using a modified version of the liquid pre-incubation assay described by Maron and Ames (21). The bacterial suspension (100 μl) and the test compound (10 μl DMSO) were added sequentially to a glass tube containing 500 μl of 100 mM MgSO4 pre-warmed to 37°C. For incubation for 60 min at 37°C, 2.0 ml of 45°C warm soft agar (5.5 mg/ml agar, 5.5 mg/ml NaCl, 50 μM biotin, 50 μM histidine, 50 μM tryptophane, 25 mM sodium phosphate buffer, pH 7.4) was added to a tube with a mixture to which 150 μl of a P. putida or E. coli minimal agar (15 mg/ml agar in Vogel-Bonner E medium with 20 mg/ml glucose). After incubation for 3 days in the dark, the colonies (his reverants) were counted. Incubations were carried out in triplicate or quadruplicate, except for the negative controls (usually twice as many plates), unless specified otherwise. Most compounds were tested in various strains on several occasions. In general, the results of the last experiment, with optimised dose levels, are presented. The result of an individual experiment was classified positive, if the number of revertants (mean value at any dose level) was increased at least 2-fold above the number of spontaneous revertant colonies with a plausible dose–response relationship. It was also classified positive if the increase was at least 1.5-fold and confirmed in repeated experiments. Specific mutagenicities (revertants per nanomole) were calculated as the slope of the initial part of the dose–response curve of the positive results. For negative results, a conservative limit of detection is given by dividing the number of spontaneous revertant colonies by the highest dose that could be adequately tested (no obvious toxicity).

Analysis of adducts formed in herring sperm DNA and in bacteria

Herring sperm DNA (20 mg, dissolved in 8 ml water) and (+)-1′-acetoxymethyleugenol (0.1 mM, dissolved in 2 ml DMSO) were incubated at 37°C for 1 h. Then, 1 ml of a sodium acetate solution (3 M, pH 5.2) was added. The DNA was recovered by precipitation with 16.5 ml ice-cold 2-ethoxyethanol and subsequent storage at −20°C for 1 h. Then, it was centrifuged at 5000 × g for 30 min, washed with 1 ml 70% ethanol, dried and dissolved in 1 ml water. The DNA concentration was determined spectrophotometrically with a Nanodrop ND-1000 spectrophotometer (peqlab Biotechnologie, Erlangen, Germany) measuring the absorbance at 260 nm.

Bacteria were exposed to the test compounds as in the mutagenicity assay. After the 60-min exposure period, they were harvested by centrifugation (10 min, 9000 × g, 4°C). The resulting bacterial pellet was washed by resuspension in 1 ml water and repeat of the dichotomy of the test step. DNA was extracted from the bacteria as described previously (22) and isolated using a standard phenol–chloroform protocol (23).

An aliquot of DNA (12.5 μg dissolved in 56 μl water), internal standards (268 fmol[15N3]-N5-MIE-dA and 156 fmol[15N2]-N5-MIE-dG in 15 μl of 75% methanol:25% water), 16 μl buffer (100 mM sodium succinate buffer, pH 6.0, 50 mM CaCl2) and 24 μl enzyme mixture (containing 2.7 μg mouse liver cytosolic N5-MIE-dG aflatoxin B1 77°C for 17°C for 16 h. Then, 38 μl of Tris buffer (0.5 M, pH 10.9) and 3 μl alkaline phosphate solution (3 U) were added and the incubation was continued over night.
Proteins were precipitated by addition of 500 µl ice-cold ethanol. After mixing and centrifugation (10 min, 19 000 × g, 4 °C), the supernatant was collected followed by removal of the solvent under reduced pressure in a Speedvac. The residue was taken up in 50 µl of 75% methanol and centrifuged (10 min, 19 000 × g, 4 °C). The supernatant was analysed using an Acquity UPLC connected sequentially to an Acquity photodiode array detector (irrelevant for the adduct analyses) and a Xevo TQ triple quadruple mass spectrometer (Waters). It was equipped with an Acquity UPLC BEH Phenyl column (2.1 × 100 mm, 1.7 µm).

A gradient of water acidified with 0.25% acetic acid (v/v) and 0.25% formic acid (v/v) (solvent A) and acetonitrile acidified equally (solvent B) was used as mobile phase at a flow rate of 0.3 ml/min. After injection of 7.5 µl of the sample, chromatographic separation was achieved using a 5-min linear gradient from 20 to 80% solvent B. Mass spectrometric analyses of the adducts were performed in the positive ionisation mode using multiple reaction monitoring (MRM) for specific detection. The tuning parameters were set as follows: temperature of electrospray source: 110 °C; desolvation temperature: 450 °C; desolvation gas (nitrogen) flow: 950 l/h; collision gas (argon) flow: 0.23 ml/min (pressure: 3.5 × 10⁻³ mbar); dwell time per transition: 48 ms; capillary voltage: 0.9 kV. Three characteristic m/z transitions were used for each adduct (Table I).

The cone voltage and collision energy were optimised for each transition with the IntelliStart tool of the MassLynx 4.1 software (Waters) and are listed in Table I.

**Determination of the limits of detection and quantification of adducts**

N²-MIE-dA and N²-MIE-dG in DNA

Unexposed DNA (12.5 µg per sample) was digested to deoxynucleosides as described in the preceding section but without the addition of internal isotope-labelled adduct standards. Immediately before UPLC–tandem mass spectrometry (MS/MS) analysis, samples were spiked with 0 (blank), 2.5, 5, 10 or 50 fmol of each adduct, N²-MIE-dA and N²-MIE-dG. The limit of detection was defined as the adduct level giving a peak area of the quantifier that was the mean plus three times the standard deviation of the blank value from 10 separate digests of unexposed DNA. The limit of detection was defined as the adduct level giving a corresponding peak area that was the mean plus nine times the standard deviation of the blank value from 10 separate digests of unexposed DNA. Under the conditions used, limits of the detection amounted to 2 N²-MIE-dA and 6 N²-MIE-dG adducts per 10¹⁰ nucleosides. The corresponding limits of quantification were 3 N²-MIE-dA and 12 N²-MIE-dG adducts per 10¹⁰ nucleosides. Usage of higher amounts of DNA in the analyses could reduce these limits. However, this was not required for the present study.

**Results and discussion**

**Mutagenicity in SULT expressing Salmonella strains**

The benzylic ester (±)-1'-acetoxymethyleugenol is an uncharged analogue of (±)-1'-sulfoxyxymethyleugenol. Therefore, it may better penetrate cell membranes than the latter. It was prepared for the synthesis of adducts. In addition, we tested it for mutagenicity in standard Salmonella strains. It elevated the numbers of revertants from strains TA100 and TA104 up to 3-fold above the spontaneous levels, requiring doses of ~100 nmol/plate (data not shown). These strains are primarily reverted by substitution mutations (21). No mutagenicity was detected with strain TA1538, a strain that detects primarily reverted by substitution mutations (21). No mutagenicity was detected with strain TA1538, a strain that detects primarily reverted by substitution mutations (21).

No mutagenicity was detected with strain TA100-hSULT1A2 (Figures 2C and 3D), indicating that the enzyme level was equally well activated in strains TA100-hSULT1A1 and TA100-hSULT1A1*Y, indicating that the enzyme level was not limiting. This finding suggests that (E)-3'-hydroxymethylsulfoxynoegenol is a better substrate for hSULT1A1 than are the 1'-hydroxymethylmethylglutarin enantiomers of 1'-hydroxymethylmethylglutarin (Figure 3H). However, the activation was weaker than when the orthologous human enzyme was expressed at a lower level (TA100-hSULT1A1) or higher level (TA100-hSULT1A1*Y) than mSult1a1 in TA100-mSult1a1.

(E)-3'-hydroxymethylsulfoxynoegenol was not mutagenic in the parental strain TA100 but was only tested up to a maximal dose of 1000 nmol/plate (Figure 2A). It was activated by the same SULT forms as its positional isomer, 1'-hydroxymethylmethylglutarin, but with some quantitative differences. (E)-3'-hydroxymethylsulfoxynoegenol, unlike 1'-hydroxymethylmethylglutarin, was equally well activated in strains TA100-hSULT1A1 and TA100-hSULT1A1*Y, indicating that the enzyme level was not limiting. This finding suggests that (E)-3'-hydroxymethylsulfoxynoegenol is a better substrate for hSULT1A1 than are the 1'-hydroxymethylmethylglutarin enantiomers. (E)-3'-hydroxymethylsulfoxynoegenol showed higher mutagenic activity than 1'-hydroxymethylmethylglutarin in TA100-hSULT1A2 (Figures 2C and 3D), whereas the situation was reverse in TA100-hSULT1C2 (Figure 3F).

**Table I. Transitions used for MS/MS analysis of adducts N²-MIE-dA and N²-MIE-dG**

<table>
<thead>
<tr>
<th>m/z transition</th>
<th>Comment</th>
<th>Cone voltage, V</th>
<th>Collision energy, eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N²-MIE-dA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>428.1 (433.1*)</td>
<td>→ 177.2</td>
<td>Loss of da, quantifier</td>
<td>33</td>
</tr>
<tr>
<td>428.1 (433.1*)</td>
<td>→ 146.1</td>
<td>Loss of da and one methoxy group</td>
<td>33</td>
</tr>
<tr>
<td>428.1 (433.1*)</td>
<td>→ 312.2 (317.2*)</td>
<td>Loss of deoxyribose</td>
<td>33</td>
</tr>
<tr>
<td>N²-MIE-dG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>444.1 (449.1*)</td>
<td>→ 328.2 (333.2*)</td>
<td>Loss of deoxyribose, quantifier</td>
<td>22</td>
</tr>
<tr>
<td>444.1 (449.1*)</td>
<td>→ 164.1 (169.1*)</td>
<td>Loss of deoxyribose and phenethyl group</td>
<td>22</td>
</tr>
<tr>
<td>444.1 (449.1*)</td>
<td>→ 177.1</td>
<td>Loss of dG</td>
<td>22</td>
</tr>
</tbody>
</table>

*Values for isotope-labelled standards are marked with an asterisk, if different from the unlabelled adduct.
It should be noted that the increases in the number of revertants induced by hydroxylated methyleugenol metabolites were rather weak in all strains, rarely exceeding the control value by a factor of >2. Therefore, we suspect that reactive sulfo conjugates formed from these compounds are relatively strongly bacteriotoxic, overshadowing mutagenic responses.

Table II. Mutagenicity of hydroxylated metabolites of methyleugenol in the standard strain Salmonella typhimurium TA100 and in TA100-derived strains expressing various human and murine SULT forms

<table>
<thead>
<tr>
<th>Expressed SULT</th>
<th>Revertants per nanomole*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(±)-1’-Hydroxymethyleugenol</td>
</tr>
<tr>
<td>None</td>
<td>0.02b</td>
</tr>
<tr>
<td>Human 1A1</td>
<td>18b</td>
</tr>
<tr>
<td>Human 1A1</td>
<td>—</td>
</tr>
<tr>
<td>(*1Y, elevated expression)</td>
<td></td>
</tr>
<tr>
<td>Human 1A2</td>
<td>0.6b</td>
</tr>
<tr>
<td>Human 1A3</td>
<td>—</td>
</tr>
<tr>
<td>Human 1C1</td>
<td>—</td>
</tr>
<tr>
<td>Human 1C2</td>
<td>—</td>
</tr>
<tr>
<td>Human 1C3</td>
<td>—</td>
</tr>
<tr>
<td>Human 1E1</td>
<td>—</td>
</tr>
<tr>
<td>Human 2A1</td>
<td>0.03b</td>
</tr>
<tr>
<td>Human 2B1b</td>
<td>—</td>
</tr>
<tr>
<td>(*1Q, high expression)</td>
<td></td>
</tr>
<tr>
<td>Murine 1a1</td>
<td>—</td>
</tr>
<tr>
<td>Murine 1b1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

—. Not tested.

*Estimated from the initial slope of the dose–response curves. As the linear part of the curves was small with the compounds tested, the values are not very accurate (factor of 1.5 for subjectivity and 2 for inter-experimental variation). For negative results, a conservative estimate of the limit of detection is given. This limit depended on the doses used with the given test compound. In particular, racemic 1’-hydroxymethyleugenol was available in larger quantities than its individual enantiomers.

#Representative curves are shown in Figures 2 and 3.

#Our standard criterion for a positive result (at least 1.5 increase in the number of revertants above the control level) was not met. However, the increases in the number of revertants were so consistent that some activation by the expressed SULT has to be inferred.

Fig. 2. Effect of expression of human SULTs in target cells on mutagenicity of (±)-1’-hydroxymethyleugenol and (E)-3’-hydroxymethylisoeugenol in the Ames test. Open rhomboids: (±)-1’-hydroxymethyleugenol; solid triangles: (E)-3’-hydroxymethylisoeugenol. Values are mean ± SE of three to four plates (substance-treated groups) or six to eight plates (negative control) from one experiment. SE is within the symbol if no error bar is visible.
Characterisation of \(N^6\)-MIE-dA and \(N^2\)-MIE-dG

dA and dG adducts were prepared by incubation of the corresponding 2'-deoxynucleosides with (-)-1'-acetoxymethyleugenol. Mass spectra and structural formulas of the adducts as well as the putative structures of the fragments are presented in Figure 4. Additional fragments were observed when the collision energy was increased. The fragments \(m/z\) 164 and 148 have been observed with other dG and dA adducts involving bonding to exocyclic amino groups of nucleobases and appear to be characteristic for them (26,27). However, it was not possible to unambiguously identify the atoms in the deoxynucleoside and methyleugenol moieties involved in bonding. We have deduced this information from the \(^1\)H-NMR spectra using the SDBS-RIO database (National Institute of Advanced Industrial Science and Technology, Japan). The following points are particularly informative: The signal for the exocyclic amine in dA (7.31 p.p.m.) was shifted to 8.07 in the dA adduct. This signal was broad (consequence of tautomerism with \(N^1\) of purine) and small (one rather than two protons). The signals 6.26 and 6.44 (alkene group) showed a high coupling constant of 16 Hz, implying \(trans\) configuration (with lower coupling constants of 6–12 Hz being typical for \(cis\) configuration). Likewise, the signal for the exocyclic amine in dG (6.50 p.p.m.) was shifted to 6.64 in the dG adduct. This signal was broad (consequence of tautomerism with \(N^3\) of purine) and small (one rather than two protons). Again, the signals 6.24 and 6.51 (alkene group) showed high coupling constants of 16 and 17 Hz, respectively, indicating \(trans\) configuration.

When dC was incubated with (±)-1'-acetoxymethyleugenol, several minor products were formed that may represent adducts. However, the levels of these products were too small for purification. dT did not appear to form adducts with (±)-1'-acetoxymethyleugenol.

Adduct formation in cell-free (herring sperm) DNA

Herring sperm DNA was incubated with or without (blank) (±)-1'-acetoxymethyleugenol, digested to deoxynucleosides...
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Fig. 4. Structural formulas of N²-MIE-dG (left) and N⁶-MIE-dA (right) with fragmentation patterns observed by positive electrospray MS/MS collision-induced dissociation. Product ion spectra of the protonated molecule of dA and dG were recorded under the same fragmentation conditions as used for adduct quantification. The adducts were generated by incubating the corresponding deoxynucleosides with (±)-1'-acetoxymethyleugenol. Their structures were corroborated by ¹H-NMR analyses (see main text), using the numbering system indicated in the structural formulas.

and analysed by UPLC–MS/MS in the MRM mode for the presence of adducts with the various nucleosides (Figure 5). A product with the retention time (2.39 min) and all three m/z transitions of N²-MIE-dG in the same ratio as observed for the authentic standard was detected at high levels (Figure 5, traces A1–A3). Weak signals for two transitions (indicative of the loss of deoxyribose with or without concomitant loss of the phenylethyl group) were seen at a retention time of 2.05 min. They may represent a second dG adduct, e.g. involving bonding via the 1'-position of methyleugenol. Most likely, it reflects a minor adduct in DNA, as its signals were not only weaker than those of N²-MIE-dG in the MRM scan but also in selected ion recording (SIR) for the mass of the positively ionised intact adduct and UV (photodiode array) scans. The situation was different when dG, rather than DNA, was incubated with (±)-1'-acetoxymethyleugenol. Then, N²-MIE-dG as well as this uncharacterised product were formed at high levels.

Likewise, two different dA adducts appeared to be formed in the DNA (Figure 5B), although at lower levels than the dG adducts. The dominating signal (occurring at 2.68 min) corresponded to N⁶-MIE-dA with all three m/z transitions in the expected ratio. The same signals were observed at nearly 10 times lower levels at 3.00 min retention time. This second signal was also weak in SIR and UV scans. The formation of this product was enhanced when dA, rather than DNA, was incubated with (±)-1'-acetoxymethyleugenol. Then, N²-MIE-dG could be demonstrated unambiguously.

DNA adduct formation in S. typhimurium under the conditions of the mutagenicity assay

In the initial experiment, strain TA100-hSULT1A1*1Y was treated with hydroxylated methyleugenol metabolites at a low concentration (6.6 μM, equivalent to a dose of 4 nmol/plate in the mutagenicity assay). Compared to the solvent control, the number of revertants was increased 1.5- to 1.7-fold by the three isomeric test substances (Table III). Levels of N²-MIE-dG adducts were similar for all three isomers. They were 21- to 31-fold above the limit of quantification. All three m/z transitions were readily detectable, as shown for TA100-hSULT1A1*1Y bacteria treated with (±)-1'-hydroxymethyleugenol in Figure 6. N⁶-MIE-dA adducts were also detected. However, their levels only were 1.5–3% of those of the dG adduct (Table III). No adducts were detected in control strain TA100 exposed to the same dose of the test compounds and in unexposed bacteria of either strain.

In the next experiment, strain TA100-hSULT1A1*1Y was treated with varying doses of (±)-1'-hydroxymethyleugenol. Adduct levels increased continuously with the dose, but the increase was hypolinear (Figure 7), similar to the dose–response curve for mutagenicity (Figure 3C). The reasons for this deviation from linearity are not known. They may involve saturation of SULT, inactivation of SULT by reactive sulfo conjugates or bacteriotoxicity, leading to compromised synthesis of the cofactor PAPS for example.
Conclusions

We have shown in the present study that methyleugenol metabolites are mutagenic in the Ames test even at relatively low concentrations if appropriate activating enzymes, SULTs, are expressed in the target cells. Among the human enzymes, SULT1A1 showed the highest activity, followed by SULT1C2. SULT1A1 is the most abundant SULT form in the human organism. In contrast, expression of SULT1C2, up to date merely detected on the RNA level, has been observed only in few adult tissues (25). To the best of our knowledge, it has not been studied whether alkenylbenzenes and their hydroxylated metabolites pass the placenta and reach foetal tissues. If this were the case, subsequent sulfation by foetal SULT forms might induce teratogenic and transplacental carcinogenic effects. However, taken together, our findings suggest that SULT1A1 is the most important SULT form involved in the activation of methyleugenol in the human organism, at least after birth. The orthologous murine form, mSult1a1, was somewhat less efficient in the activation of methyleugenol.

Table III. Levels of DNA adducts formed by hydroxylated metabolites of methyleugenol in Salmonella typhimurium strains under the conditions of the mutagenicity assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TA100</th>
<th>TA100-hSULT1A1*1Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Revertants per plate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Adducts per 10&lt;sup&gt;8&lt;/sup&gt; nucleosides&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>N&lt;sup&gt;6&lt;/sup&gt;-MIE-dA</td>
<td>N&lt;sup&gt;2&lt;/sup&gt;-MIE-dG</td>
</tr>
<tr>
<td>Negative (solvent) control</td>
<td>113 ± 5</td>
<td>—</td>
</tr>
<tr>
<td>(−)-1′-Hydroxymethyleugenol, 4 nmol</td>
<td>117 ± 3&lt;sup&gt;™&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>(+)-1′-Hydroxymethyleugenol, 4 nmol</td>
<td>101 ± 9&lt;sup&gt;™&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>(E)-3′-Hydroxymethylisoeugenol, 4 nmol</td>
<td>111 ± 5&lt;sup&gt;™&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>
| <sup>a</sup>Mean ± SE of six plates (negative control) or five plates (treatment groups).
| <sup>b</sup>Mean ± SE of three incubations (each scaled up 10-fold over that of the mutagenicity assay).
| <sup>™</sup>below limit of detection (2 N<sup>6</sup>-MIE-dA and 6 N<sup>2</sup>-MIE-dG adducts per 10<sup>8</sup> nucleosides).

<sup>™</sup>Student’s t-test compared to negative control: ns, P > 0.2; <sup>***</sup>P < 0.001.
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metabolites. It is highly expressed in liver, gut and lungs (28).

One of these tissues, the liver, is the major target tissue for methyleugenol-induced tumorigenesis in the mouse (1,2). The liver expresses high levels of cytochromes P450, required for conversion of methyleugenol to its hydroxylated metabolites, which in turn may be further activated in liver by local SULT enzymes, including Sult1a1.

All three hydroxylated methyleugenol metabolites studied, \((-\)-1’'-hydroxymethyleugenol, \((+\)-1’'-hydroxymethyleugenol and \((E\)-3’'-hydroxymethylisoeugenol were activated to mutagens by SULTs with moderate quantitative differences. Whereas \((E\)-3’'-hydroxymethylisoeugenol was equally mutagenic in both strains expressing hSULT1A1 at different levels, the effects of both enantiomers of 1’'-hydroxymethyleugenol were enhanced with the increased expression level, suggesting that \((E\)-3’'-hydroxymethyleugenol was the better substrate. This is not particularly surprising as its hydroxyl group is located at the end of a relatively flexible side chain, which may facilitate interaction with the active centres of enzymes. In contrast, the 1’'-hydroxyl group is somewhat sterically hindered. Whereas \((\pm\)-1’'-hydroxymethyleugenol has demonstrated higher carcinogenic activity in mouse liver than methyleugenol (1), \((E\)-3’'-hydroxymethylisoeugenol has not yet been studied for carcinogenicity. However, the corresponding metabolite of another alkenylbenzene, safrole, has been investigated for various activities. Although 1’'-hydroxysafrole and 3’'-hydroxyisosafrole formed similar levels of adducts with nucleic acids in the presence of PAPS-fortified murine hepatic cytosol, 3’'-hydroxyisosafrole was much less carcinogenic and formed much lower levels of adducts in mouse liver \textit{in vivo} than 1’'-hydroxysafrole (29). The difference was explained by the rapid biotransformation of 3’'-hydroxyisosafrole in the mouse \textit{in vivo} to the corresponding carboxylic acid. It remains to be studied whether the same sequestration occurs in humans and with 3’'-hydroxylated metabolites of other alkenylbenzenes, such as methyleugenol.

Finally, we devised a mass spectrometric method for the specific detection of DNA adducts formed by methyleugenol. The usage of isotope-labelled internal standards allows accurate quantification. All three hydroxylated methyleugenol metabolites studied formed the same DNA adducts, high levels of \(N_2\)-MIE-dG and \(\approx 50\) times lower levels of \(N_6\)-MIE-dA in SULT-expressing \textit{S. typhimurium} under the conditions of the mutagenicity assay. The sensitivity of the method is demonstrated by the observation that the level of \(N_2\)-MIE-dG adducts were \(21\) to \(31\)-fold above the limit of quantification at doses of the test compounds leading to \(1.5\) to \(1.7\)-fold increases in the number of revertants. Since the method is specific for these particular adducts, it will be useful when studying methyleugenol DNA adducts in tissue and blood samples of human subjects potentially exposed to many different genotoxicants.

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Fig. 6. Unambiguous detection of \(N^2\)-MIE-dG adducts in \textit{Salmonella typhimurium} TA100-hSULT1A1*1Y treated with \((\pm\)-1’'-hydroxymethyleugenol under the conditions of the mutagenicity assay. Strains TA100-hSULT1A1*1Y (panel A) and TA100 (panel B) were exposed to 4 nmol \((\pm\)-1’'-hydroxymethyleugenol per incubation (610 \(\mu\)l). DNA was spiked with isotope-labelled internal standards, digested to deoxynucleosides and subjected to UPLC–MS/MS analyses in the MRM mode. Traces A1–3 and B1–3 represent characteristic \(m/z\) transitions of the analyte (see Table I). Traces A4 and B4 represent the transition of the quantifier for the internal standard.

Fig. 7. Dose–response curve for DNA adducts formed by \((\pm\)-1’'-hydroxymethyleugenol in \textit{Salmonella typhimurium} TA100-hSULT1A1*1Y under the conditions of the mutagenicity assay. Upper panel: \(N^2\)-MIE-dG adducts; lower panel: \(N^6\)-MIE-dA adducts. Values are mean \pm SE of three incubations. Mutagenicity results are presented in Figure 3C.
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References


