Estragole: DNA adduct formation in primary rat hepatocytes and genotoxic potential in HepG2-CYP1A2 cells

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Abbreviations: CYP, cytochrome P450; dA, desoxyadenosine; DAPI, 4′,6-diamidino-2-phenylindole; dG, desoxyguansosine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetate; ESI, FCS, fetal calf serum; gpt, xanthine-guanine phosphoribosyl transferase; HPLC, high performance liquid chromatography; LC, liquid chromatography; LOQ, limit of quantification; MS, mass spectrometry; NMR nuclear magnetic resonance; PBS, phosphate-buffered saline; pRH, primary rat hepatocytes; RNAase, ribonuclease; RP 18, reversed phase C_{18}; SDS, sodium dodecylsulfate; SP_1, specificity protein 1; SULT, sulfotransferase; TRIS, tris(hydroxymethyl) aminomethane; UDS, unscheduled DNA synthesis; UHPLC, ultra-high performance liquid chromatography; UV/VIS, ultra-violet/visible.

Abstract

Estragole is a natural constituent in herbs and spices and in products thereof such as essential oils or herbal teas. After cytochrome P450-catalyzed hydroxylation and subsequent sulfation, estragole acts as a genotoxic hepatocarcinogen forming DNA adducts in rodent liver. Because of the genotoxic mode of action and the widespread occurrence in food and phytomedicines a refined risk assessment for estragole is needed. We analyzed the time- and concentration-dependent levels of the DNA adducts N^2-(isoestragole-3‘-yl)-2‘-desoxyguanosine (E3’N^2dG) and N^6-(isoestragole-3‘-yl)-desoxyadenosine (E3’N^6dA), reported to be the major adducts formed in rat liver, in rat hepatocytes (pRH) in primary culture after incubation with estragole. DNA adduct levels were measured via UHPLC-ESI-MS/MS using stable isotope dilution analysis. Both adducts were formed in pRH and could already be quantified after an incubation time of 1 h (E3’N^6dA at 10 µM, E3’N^2dG at 1µM estragole). E3’N^2dG, the main adduct at all incubation times and concentrations, could be detected at estragole concentrations $\leq$ 0.1 µM after 24 h and $\leq$ 0.5 µM after 48h. Adduct levels were highest after 6 h and showed a downward trend at later time-points, possibly due to DNA repair and/or apoptosis. While the concentration-response characteristics of adduct formation were apparently linear over the whole concentration range, strong indication for marked hypo-linearity was obtained when the modeling was based on concentrations $\leq$ 1 µM only. In the micronucleus assay no mutagenic potential of estragole was found in HepG2 cells whereas in HepG2-CYP1A2 cells 1 µM estragole led to a 3.2 fold and 300 µM to a 7.1 fold increase in micronuclei counts. Our findings suggest the existence of a ‘practical threshold’ dose for DNA adduct formation as an initiating key event of the carcinogenicity of estragole indicating that the default assumption of concentration-response-linearity is questionable, at least for the two major adducts studied here.

1. Introduction

The allylic phenylpropanoid estragole is a natural constituent in herbs and spices such as anise basil, fennel, tarragon etc. (Atkinson, 2018). After oral exposure, estragole is readily absorbed from the gastrointestinal tract and can undergo metabolism mainly in the liver (Smith et al. 2002). Among several metabolic pathways, there is a bioactivating pathway via 1'-hydroxylation at the allylic side-chain with subsequent enzymatic sulfation. The semi-stable, reactive sulfate conjugate thus formed can undergo sulfate elimination under formation of covalent bonds of the remaining moiety to nucleophilic targets (Miller et al., 1983). The major enzymes catalyzing the first critical step (1'-hydroxylation) are cytochromes P450 (CYP), most notably CYP1A2 (Jeurissen et al., 2007), and for the phase II step (sulfation of 1'hydroxyestragole), sulfotransferases, most notably SULT1A1 (Suzuki et al., 2012b).

There is accumulating evidence that the 1'-sulfoxy metabolite of estragole can also react with DNA leading to covalent adducts with guanine or adenine (Phillips et al., 1981). These are considered as pro-mutagenic lesions and major contributors to the genotoxicity and carcinogenicity of estragole observed in laboratory rodents (Miller et al., 1983).

In general, the family of allylic phenyl propanoides has been investigated by several authors for its capacity to form DNA adducts in the liver. Examples are methyleugenol (Herrmann et al., 2014), ß-asarone (Stegmüller et al., 2018) and estragole (Phillips et al., 1981).

Genotoxicity research on estragole including the search for DNA adducts has been focused on the liver, the principal target organ of carcinogenicity in mice (Drinkwater et al., 1976) and probably also in rats (Suzuki et al., 2012a). DNA adduct structures reported or suggested by Phillips et al. (1981) to be formed in mouse liver *in vivo* upon treatment of the animals with 1'hydroxyestragole are N^2-(estragole-1′-yl)-2′-desoxyguanosine (E1′N^2dG), the two diastereomeric adducts N^2-(trans-isoestragole-3′-yl)-2′-desoxyguanosine (trans-iE3'N^2dG) and N^2-(cis-isoestragole-3′-yl)-2′-desoxyaguanosine (cis-iE3′N^2dG) and the adenosine adduct (N^6-(isoestragole-3′-yl)-2′-desoxyadenosine (iE3′N^6dA). In addition Wiseman et al. (1985) found

C8-(isoestragol-3'-yl)-2'- desoxyguanosine (C8iE3'dG) in a reaction of 1'-acetoxyestragole with desoxyguanosine. Using a similar approach, Punt et al. (2007) found the adducts C8iE3'dG, two diastereomers of E1′N^2dG, and trans-iE3'N^2dG, the latter being the major adduct found in incubations of 1'-hydroxyestragole with hepatic PAPS-fortified S9-mix from human, rat or mouse. In rats treated with estragole over four weeks, trans-iE3'N^2dG trans-iE3′N^6-dA, and C8iE3'dG were found in the liver, iE3'N^2dG and iE3′N^6-dA being the major adducts (Ishii et al., 2011). Suzuki et al. (2012b) found a dose-dependent increase in the same estragole-derived hepatic DNA adducts after treatment of mice with estragole. Furthermore, at a dose level of 250 mg/kg b.w., significant increases in hepatic mutations in the SP1- and the gpt-locus were found in females. In rats, estragole treatment also led to the formation of DNA adducts in the liver and increased mutation frequencies at the gpt-locus with doses of 200 and 600 mg/kg b.w. At lower dose levels, no significant mutagenicity was found (Suzuki et al., 2012a).

Nesslany et al. (2010) found increased unscheduled DNA synthesis (UDS) in hepatocytes of male rats 12-16 h after treatment (by gavage) with estragole at 250 mg/kg b.w. and at higher doses. With tarragon leaves they could not see any effects (up to the maximum dose of 6.25 g/kg b.w. (18.75 mg estragole/kg b.w.).

Here, we present data on the levels of two major DNA adducts, reported to be formed in rat liver, in estragole-treated pRH with a special focus on concentration-response characteristics. Furthermore, we show results on micronuclei formation in human HepG2 hepatoma cells over-expressing human CYP1A2, an enzyme considered as the major contributor to the 1'-hydroxylation of estragole as the initiating metabolic step leading to genotoxicity and carcinogenicity.

2. Materials and methods

2.1. Animals, cells and materials

2'-Desoxyguanosine (dG), 2'-desoxyadenosine (dA), 2'-desoxycytosine were from AppliChem GmbH (Darmstadt, Germany) or TCI (Eschborn, Germany). The isotope-labeled nucleosides $^{15}N_5$-dG and $^{15}N_5$-dA with a degree of labeling of > 98 % were purchased from Silantes GmbH (Munich, Germany). Methanol (HPLC grade) was from VWR (Darmstadt, Germany), water was double distilled after deionization. RNAse was from Carl Roth (Karlsruhe, Germany), proteinase K and alkaline phosphatase from Sigma-Aldrich (Taufkirchen, Germany), phosphodiesterase and micrococcal nuclease from Worthington (Lakewood, NJ, USA). All other chemicals including solvents were at the highest purity commercially available from major suppliers. All plastic labware was from Greiner Bio-One (Frickenhausen, Germany).

HepG2 cells were obtained from DSMZ (Heidelberg, Germany), HepG2 cells transfected with the human CYP1A2 gene (HepG2/CYP 1A2 cells, C7) were generated as described (Steinbrecht et al., 2020). pRH were isolated from young adult male Wistar rats, obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animals were kept, and pRH were isolated according to the German Animal Protection Act and under the control of and with permission from the responsible authorities of the State of Rhineland-Palatinate, Germany.

2.2. Synthesis and identification of estragole-derived DNA adducts

N^6-(isoestragole-3'-yl)-desoxyadenosine (iE3'N^6dA): 2′-Desoxyadenosine (100 mg; 371 µmol) was diluted in 47.5 mL aqueous ammonium carbonate solution (10 mM). 1′-acetoxyestragole (192 mg; 929 µmol) was diluted in 2.5 mL acetonitrile. Both solution were mixed. The reaction mixture was stirred for 72 h at 37 °C and subsequently separated via preparative HPLC. The solvent of the combined product containing fractions was removed via lyophilisation and the compounds were further dried in vacuo. Yield: 2 %. Analysis: t_R

(HPLC) 24.8 min; t_R (UHPLC) 3,71 min; LC-MS/MS fragmentation: $[M+H]^+$ 398.2 m/z, [M-desoxyribose]$^+$ 282.8m/z, [M-dG]$^+$ 147.1; ^{1}H NMR (600 MHz, DMSO-d6) δ [ppm]: 2.25 (m, 1 H, 2′dR); 2.72 (m, 1 H, 2′dR); 3.51 (m, 1 H, 3'R); 3.61 (m, 2 H, 3'R); 3.69 (m, 1 H, 5′dR); 3.72 (s, 3 H, OCH_3); 3.86 (m, 1 H, 5′dR); 4.24 (s, 1 H, OH); 4.40 (s, 1 H, OH); 5.22 (s, 1 H, 3′dR); 5.32 (m, 1 H, 4′dR); 6.21(m, 1 H, 1′dR); 6.34 (m, 1 H, 2′R); 6.45 (m, 1 H, 1′R); 6.85 (m, 2 H, HAr); 7.32 (m, 2 H, HAr); 8.52 (m, 1 H, NH); 8.20 (s, 1 H, C2-dA); 8.35 (s, 1 H, C8-dA).

N^2-(isoestragole-3'-yl)-2'-desoxyguanosine (iE3'N^2dG): 2′-Desoxyguanosine (100 mg; 351 µmol) was diluted in 47.5 mL aqueous ammonium carbonate solution (10 mM). 1′-acetoxyestragole (180 mg; 870 µmol) was diluted in 2.5 mL acetonitrile. Both solution was mixed. The reaction mixture was stirred for 72 h at 37 °C and subsequently separated via preparative HPLC. The solvent of the combined product containing fractions was removed via lyophilisation and the compounds were further dried in vacuo. Yield: 7.4 %. Analysis: t_R (HPLC) 22.0 min; t_R (UHPLC) 3,17 min; LC-MS/MS fragmentation: $[M+H]^+$ 414.3 m/z, [M-desoxyribose]$^+$ 298.1 m/z, [M-dG]$^+$ 147.1, [M-desoxyribose+NH_3]$^+$ 164,1; ^{1}H NMR (600 MHz, DMSO-d6) δ [ppm]: 2.12 (m, 1 H, 2′dR); 2.85 (m, 1 H, 2′dR); 3.48 (m, 1 H, 3'R); 3.55 (m, 2 H, 3'R); 3.72 (s, 3 H, OCH_3); 3.73 (s, 1 H, 5'dR); 3.79 (s, 1 H, 5'dR); 4.35 (s, 1 H, OH); 4.88 (s, 1 H, OH); 5.28 (s, 1 H, 3′dR); 5.53 (m, 1 H, 4′dR); 6.10 (m, 1 H, 1′dR); 6.81 (m, 1 H, 2′R); 6.90 (m, 1 H, 1′R); 6.87 (d, J= 8.44, 1 H, HAr); 6.90 (d, J= 8.44, 1 H, HAr); 7.26 (d, J= 8.44, 1 H, HAr); 7.33 (d, J= 8,80, 1 H, HAr); 7.6 (s, 1 H, C8-dG); 8.52 (m, 1 H, NH).

Isotope-labeled standards: $^{15}N_5$-dG (1.121 mg; 4.12 µmol) or $^{15}N_5$-dA (1.046 mg, 4.08 µmol) were used to synthesize isotope-labeled internal adduct standards with mg 7.98 and 7.98 mg 1′-acetoxyestragole, respectively, in a total volume of 500 µl. Therefore $^{15}N_5$-dG or $^{15}N_5$-dA weighed directly into a 1.5 mL vial and solved in 440 µL aqueous ammonium carbonate solution (10 mM) and 1′-acetoxyestragole was solved in 60 µL acetonitrile. After vigorously

mixing of both solutions the reaction mixture was stirred over 72 h at 37 °C. Separation was accomplished by preparative HPLC. The solvent of the combined product containing fractions were reduced to in a speed vac. The concentration of the yielded isotope-labeled standard solutions was determined via HPLC-UV/VIS using known concentrations of the corresponding unlabeled derivatives. Yields: [$^{15}N_5$]-E3'N^2dG: 1 %); [^{15}N5]-(E3'N^6dA) 13.1 %. Analysis: LC-MS/MS: [$^{15}N_5$]-E3'N^2dG: $[M-H]^+$ 419.051 m/z $[M\text{-desoxyribose}]^+$ 298.100 m/z, $[M\text{-}^{15}N_5dG]^+$ 147.000 m/z. [$^{15}N_5$]-E3'N^6dA: $[M-H]^+$ 403.227 m/z $[M\text{-desoxyribose}]^+$ 287.200 m/z, $[M\text{-}^{15}N_5dA]^+$ 147.200 m/z.

2.3. NMR spectroscopy

NMR spectra were recorded using a Bruker (Bruker BioSpin, Rheinstetten, Germany) Avance 600 MHz spectrometer at room temperature, the test compound being dissolved in DMSO-d6 (1H NMR: δ 2.49 ppm). The data were processed using 1D Win NMR software (Bruker BinSpin, Rheinstetten, Germany).

2.4. Preparative HPLC

For separation an Agilent 1200 Series HPLC (Agilent Technologies, Kronberg, Germany) system equipped with two preparative pumps (G1361A), an automatic fraction collector (G1364B), and a *Multi-Wavelength* detector (MWD, MWD G1315A). As liquid phases double distilled water (A) and HPLC-grade methanol (B) or acetonitrile (C) were used. Data were analyzed using ChemStation software for LC (version B04.01, Agilent Technologies, Waldkirch, Germany). It was used an RP18 column (VDSpher PUR C18-SE, 5 μm, 250×20 mm, VDS Optilab, Berlin, Germany).

After injecting 10 ml sample volume, separation was carried out as follows (switch time/% B). For the first purification step of $^{15}N_5$-E3'N^2dG: 0 min/40%, 50 min/90%, 60 min/40% (flow rate 3 ml/min over 5 min, 15 ml/min over 55 min); for the second purification step of

$^{15}N_5$-E3'N^2dG: 0 min/1%, 65 min/50%, 70 min/80%, 76 min/95%, 87 min/1% (15 ml/min throughout); for the separation of unlabeled E3'N^2-dG: 0 min/40%, 50 min/90 %, 60 min/40% (3 ml/min over 5min, 15 ml/min over 55 min); for the purification of $^{15}N_5$-E3'N^6dA: 0 min/20 %, 50 min/80 %, 60 min/20% (flow rate 2 ml/min over 5 min, 15 ml/min over 55 min); for the separation of unlabeled E3'N^6dA: 0 min/20%, 50 min/80%, 60 min/20 % (3 ml/min over 5 min, 15 ml/min over 55 min).

2.5. Analytical HPLC

The system consisted of an Agilent 1200 Series HPLC unit (Agilent Technologies, Kronberg, Germany), equipped with a G1322A degasser, a G1311A pump, a G1329A autosampler, a G1316A column oven, and a G1315A diode array detector. The system was run with a ChemStation for LC software (version B04.01, Agilent Technologies, Kronberg, Germany). As liquid phases 0.1 % aqueous formic acid (A), methanol (B) were used. After injection of a sample volume of 50 µl, the following gradients were used to monitor the reaction between dA/dG and 1'-acetoxyestragole or 3'-acetoxyanethol, respectively (switch time/% B): 0 min /1%, 20 min/70%, 25.1 min/95%, 30.1 min/1% (flow rate 1 ml/min at 25°C); for the separation of $^{15}N_5$E3'N^2-dG (switch time/% B): 0 min/1%, 20 min/50%, 21 min/80%, 29 min/85%, 24.1 min/1% (flow rate 1 ml/min at 25°C).

An RP18 column (LiChrospher®, 5 µm, 4.0 mm×4.0 mm, Merck, Darmstadt, Germany) with a pre-column filled with the same stationary phase was used.

2.6. Quantification of DNA adducts (LC-MS/MS)

Samples were separated on an Agilent 1290 Series UHPLC System (Agilent Technologies, Kronberg, Germany), equipped with a 6133A degasser, a G4220A pump, a G1367C autosampler, and a G1316C column oven. A UHPLC column filled with RP18 as stationary phase (U-VDSpher PUR C18-E 1.8 µm; 50×4.6 mm) and a pre-column filled with the same

material were used. As liquid phases 0.1% aqueous acetic acid (A) and methanol with 1 % acetic acid (B) were used at a flow rate of 0.8 ml/min at 25° C. Separation was achieved with a gradient with (switching time/% B) 0 min/10%, 1.2 min/50%, 4.3 min 90%, 4.31 min/95%, 6.01 min/10 % at a flow rate of 0.8 ml/min. The injection volume was 5 µl.

The system was coupled to a Sciex QTrap 5500 MS mass spectrometer AB Sciex, Darmstadt, Germany). Measurements were run using Analyst software 1.7 (AB Sciex, Darmstadt, Germany), and data analysis with Multiquant software 2.0 AB Sciex, Darmstadt, Germany).Ionization was achieved with an electron spray in the positive mode (ESI+) using *Multiple Reaction Monitoring* (MRM). Instrument specific parameters were the following: ion spray voltage 5.0 kV; ion source temperature 500 °C; curtain gas 45 psi; nebulizer gas 55 psi; heater gas 50 psi; dwell time 50 msec.

2.7.Quantification of desoxyguanosine (dG)

The dG concentrations in the hydrolysates were determined using HPLC/MS-MS methodology. The system consisted of an Agilent 1200 series equipped with 6133A degasser, a G1367C autosampler, a G1213B pump, and a G1316B column oven (Agilent, Waldkirch, Germany). Separation was carried out on a RP18 column (LiChrospher®, 5µm, 4.0mm x 125 mm, Merck, Darmstadt, Germany) and a corresponding pre-column filled with the same stationary phase. As liquid phases 0.1% aqueous formic acid (A) and methanol (B) were used. Separation was achieved with a gradient with (switching time/ % B) 0 min/1%, 7 min/70%, 12.1 min 95%, 17.1 min/1%, at a flow rate of 0.7 ml/min at 20°C. The injection volume was 2.5 µl.

The column was coupled to a TriplQuad mass spectrometer API3200 (AB Sciex, Darmstadt, Germany)) using Analyst Software 1.4.2 (Applied Biosystem, MDS Sciex, USA). For data analysis, the Multiquant Software 2.0 (AB Sciex, Darmstadt, Germany)) was used. Ionization was achieved with an electron spray in the positive mode (ESI+) using *Multiple Reaction*

Monitoring (MRM). Instrument specific parameters were the following: ion spray voltage 4.5 kV; ion source temperature 450 °C; curtain gas 50 psi; nebulizer gas 45 psi; heater gas 50 psi; dwell time 100 msec.

2.8. Cell culture

Primary rat hepatocytes (pRH) were isolated in a two-step perfusion method (Schrenk et al., 1992). Cell preparations with a vitality > 95 % were seeded on collagenated 100 mm Petri dishes for adduct analysis (7 x 10^6 cells/7 ml) or on 24 well plates for cytotoxicity measurement (2,5 x 10^5 cells/1 ml) in DMEM (low glucose, Life Technologies GmbH, Darmstadt, Germany) with 10% FCS (Life Technologies GmbH, Darmstadt, Germany) und 1% Penicillin/Streptomycin-solution (Sigma-Aldrich, Taufkirchen, Germany). After 3-4 h, medium was replaced by fresh medium and the cells were incubated over 1 – 48 h with the test substances (dissolved in DMSO) or with 0.1 % DMSO only (negative control). 0.1 % saponin (final concentration, dissolved in water) was used as positive control for cytotoxicity testing. HepG2/CYP 1A2 (C7) cells were cultivated exactly as previously described (Steinbrecht et al., 2020).

2.9. DNA extraction and hydrolysis, sample preparation

After incubation, cells were washed with PBS and kept at -80°C until further processing. Therefore, the cells were lysed on ice with 800 µl lysis buffer (35 mM TRIS, 0.56 mM NaEDTA, 0.018 nM formic acid, 1 % SDS and 0.5 % TritonX100) and transferred into a 2 ml test tube. Then, 15 µl proteinase K (10 mg/ml) and 5 µl RNAse (10 mg/ml) were added and the mixture was kept for 3 - 4 h at 55 °C. After cooling to room temperature, 800 µl extraction solution I (phenol:chloroform:isoamyl alcohol, 25:24:1) were added and vortexed. The lysates were then centrifugated at 13,000 x g and 4°C for 10 min. The supernatants were incubated with 5 µl RNase (room temperature, 20 min), and were then treated with 700 µl extraction

solution II (chloroform:isoamyl alcohol, 24:1). After vortexing, the suspensions were centrifugated at 13,000 x g and 4°C for 10 min. The supernatants were removed and DNA was precipitated by adding 1.2 ml cold ethanol (100 %). After centrifugation at 13,000 x g and 4°C over 10 min, the pellets were dissolved in 250 µl water and 25 µl 3 M sodium acetate solution, and were DNA was precipitated with 500 µl isopropanol. After centrifugation at 13,000 x g and 4°C over 10 min, the pellet was re-suspended in 700 µl aqueous ethanol (70 %) and vortexed. After centrifugation (10 min at 4 °C and 13,000 x g), the pellet was dried at room temperature and dissolved in 50 µl water. The purity of the DNA was analyzed spectrophotometrically with a Nanodrop ND-1000 (ThermoScientific, Wilmington, NC, USA), and the DNA content was adjusted to 30 µg per sample. Each sample was spiked with 50 fmol isotope-labeled adducts and 1 nmol $^{15}N_5$-dG , an DNA was isolated according to Schumacher et al. (2013).

2.10.Cytotoxicity testing

pRH were seeded into collagenated 24 well plates (2,5 x 10^5 cells/ml) with 1 ml/well DMEM low glucose with 10% FCS and 1% penicillin/streptomycin solution over 3-4 h. HepG2 cells were seeded into 24 well plates (1.5 x 10^5 cells/ml) in 1 ml/well DMEM high glucose with sodium pyruvate, 10% FCS and 1% penicillin/streptomycin solution over 24 h, HepG2/CYP1A2 cells were cultured under the same conditions except for medium without added sodium pyruvate.

Cultures were incubated over 24 h or 24 h and further cultivation for 72 h without test compound (HepG2 and HepG2/CYP1A2 cells) or 24 h (pRH) with the test substances dissolved in DMSO (0.1 % final concentration), DMSO only served as negative control, 0.1% saponin (final concentration) as positive control. After washing with PBS, 1 ml 44 µM resazurin solution in pure medium was added per well. After incubation over 60 min (HepG2 and HepG2/CYP1A2 cells) or 90 min (pRH) with resazurin solution, fluorescence was

measured in a Fluoroscan plate reader at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

2.11. Micronucleus assay

HepG2 or HepG2/CYP1A2 cells were seeded on 60 mm Petri dishes in DMEM/high glucose (HepG2) or low glucose (HepG2/CYP1A2)/FCS/Pen-Strep at a density of 7 x 10^5 cells/2 ml medium. After 24 h medium was replaced by fresh medium and the cells were incubated with the test compounds dissolved in DMSO (0.1 % final concentration) over 72 h. Negative controls were treated with DMSO only, 0.5 µM mitomycin C (added as solution in DMSO) served as positive control. Then, the cells were trypsinized and the suspensions were centrifugated 500 x g for 5 min. The pellet was re-suspended in 1 ml PBS plus 3 ml ice-cold ethanol and vortexed. After incubation at -20 °C over 30 min, the suspension was centrifugated at 1,000 x g for 10 min. The supernatant was discarded and the pellet was stored at -20°C. For micronuclei counting, the pellet was re-suspended in 750 µl DAPI dye solution (13.8 µg/ml aqueous DAPI solution), and vortexed. After incubation at room temperature in the dark over 15 min, 10 µl of the suspension were transferred to slides and covered with cover slips (50 x 24 mm). After adding immersion oil on the cover slip, 1,000 nuclei were inspected under a fluorescence microscope (Axioskop, Zeiss, Jena Germany) at 64 x magnification. Micronuclei were identified as round or oval-shaped bodies with a diameter of 1/3 of the corresponding nucleus at maximum, being stained more intensively as the nucleus, laying adjacent to a nucleus, not showing ‘bridging’ to a nucleus, being in same optical focus as the corresponding nucleus.

2.12. Statistical analysis and modeling

Data represent means ± S.D. from n=3 independent experiments. For multiple comparison with a control, Dunnett’s test was applied with probabilities of error (p) as indicated. For

concentration-response modeling, threshold ~~doses~~ concentrations (tc) were determined using the broken stick model (Lutz and Lutz, 2009) in R for windows version 3.6.3 (R Core team, 2020).

3. Results

The synthesis of unlabeled and isotope-labeled N^2-(isoestragole-3‘-yl)-2‘-desoxyguanosine (E3’N^2dG) and N^6-(isoestragole-3‘-yl)-desoxyadenosine (E3’N^6dA) led to the expected adducts as demonstrated by mass (Figs. 1 A and B) and ^{1}H-NMR spectroscopy. The reference compounds were used as calibration standards for the quantitative analysis of both adducts in pRH.

In pRH, estragole led to a slight non-significant decrease in viability (data not shown) whereas HepG2-CYP1A2 cells were more sensitive with a significant loss in viability at 30 µM and above (Fig. 2). These data point to differences in handling of the substrate including uptake and metabolic activation and/or inactivation. The loss of viability in HepG2-CYP1A2 cells did not exceed 50 %, a condition for the micronuclei assay (OECD, 2016). Non-transfected HepG2 cells showed no estragole toxicity as expected due to the lack of critical CYP activities.

There, incubation with increasing estragole concentrations led to a time- and concentration-dependent increase in DNA adducts. Both adducts were found in an approximate ratio of dA:dG of 1:10. Significant levels of adducts were found already after 6 h at $\geq$ 10 µM and after 12 h at > 1 µM estragole. The time course of adduct levels was relatively similar between the dA (Fig. 3A) and dG (Fig. 3B) adduct. At estragole concentrations above 10 µM, the highest levels were seen after 12 h and a decrease by about 10 – 40 % after 48 h. Interestingly, the dG adduct level decreased more effectively over time than the dA adduct level. However, this time course was seen only for substrate concentrations $\geq$100 µM while at lower substrate concentrations levels remained almost constant or even increased between 6 and 48 h.

In a next step a concentration-response analysis for adduct levels was carried out to gain more information about the underlying mathematical function and key parameters. For this purpose, the data were modeled against a broken stick, which reveals a threshold concentration (tc) +/- 95% confidence intervals. Should the intervals have bridged zero, the response was considered linear. Given the protracted ~~dose~~ concentration range, and the subsequent compression of the lower ~~doses~~ concentrations (apparent in Fig. 4A), it was considered judicious to model both the entire ~~dose~~ concentration-range and the lower ~~doses~~ concentrations alone, in case potential tcs, at the lower concentrations, were being masked by this compression.

The analysis of the dA adduct revealed a linear relationship between the substrate concentration and the adduct level if the total concentration range was considered (Fig. 4A). When the calculation was focused on the three lowest estragole concentrations ($\leq$ 1 μM), however, a non-linear function became obvious (Fig. 4B) which allowed the estimation of a 'practical threshold' at a concentration of about 0.55 μM (95% confidence interval, rounded: 0.46 - 0.99 μM). Below this concentration no increase in adduct levels above the LOQ and no effect of estragole on the adduct level could be observed. For the dG adduct a very similar picture was obtained for all concentrations (Fig. 4C) and for the lowest concentration range at $\leq$ 1 μM (Fig. 4D). From these data a practical threshold of about 0.47 μM (95% confidence interval, rounded: 0.43 - 0.99 μM) could be derived. It needs to be mentioned that this type of influence of the concentration range was obtained for all time points analyzed, i.e. after 1, 6, 24 and 48 h of treatment for the dG adduct and after 6, 24, and 48 h for the dA adduct (after 1 h, no dA adducts were found at estragole concentrations $\leq$ 1 μM). The calculated threshold concentrations were relatively independent from the time point, i.e. were at 0.5 μM as a mean value (Table 2).

In order to analyze the genotoxic consequences related to estragole exposure, we next analyzed the levels of micronuclei in HepG2-CYP1A2 cells. Estragole concentrations

between 1 and 300 μM led to a concentration-dependent increase in micronuclei counts. At the lowest concentration of 1 μM the number was already significantly increased when compared to the control (Fig. 5). Testing of lower concentrations did not lead to increases over the control rate. However, the large variability between measurements in this concentration range (data not shown) did not warrant a concentration-response modeling.

4. Discussion

Estragole, a natural constituent in a variety of herbs and spices used in food and/or phytomedicine, has drawn much attention with respect to its hepatotoxic and carcinogenic properties (Martins et al., 2018). Thus, there is an increasing need for a thorough assessment of the risk of human estragole exposure with a special emphasis on the mode(s) of action of the compound and the dose (concentration) - response characteristics. Here, we investigated cytotoxicity and DNA adduct levels in estragole-treated rat pRH as a prototype target cell type for estragole toxicity. As a suitable human cell model for genotoxicity and cytotoxicity testing of estragole, metabolically competent HepG2-CYP1A2 cells were used.

In previous studies, postnatal treatment of mice with estragole or 1'-hydroxyestragole significantly increased the incidences of hepatoma and hepatocellular carcinoma in the adult animals (Drinkwater et al., 1976; Miller et al., 1983). The affected livers also showed various types of non-neoplastic lesions such as chronic inflammation, portal fibrosis, bile duct proliferation, and focal areas of atypical cellular hyperplasia and megalocytosis. In rats, the evidence for carcinogenicity of estragole is less clear. At relatively high dose levels (75-600 mg/kg b.w. per day), pre-neoplastic lesions such as hepatocellular hypertrophy, bile duct and oval cell hyperplasia, cholangiofibrosis, and chronic periportal inflammation were observed (NTP, 2011). Auerbach et al. (2010) reported changes in hepatic gene expression in estragole-treated rats, indicating that estragole, also in rats, should be classified as a hepatocarcinogen.

In our study, estragole did not exert significant cytotoxicity in pRH over an incubation period

of 24 h. Longer incubation periods were not used because of the substantial loss of drug-metabolizing enzyme activities in pRH cultured over longer time (Gao et al., 2020). In contrast, human HepG2-CYP1A2 cells showed some cytotoxicity at estragole concentrations $\geq$ 30 µM after 24 h incubation plus 72 h recovery, a schedule also used for the micronuclei assay. It has to be noted that none of the concentrations led to a loss in vitality exceeding 50 % as recommended by the OECD as a condition for micronuclei analysis (OECD, 2016). In non-transformed HepG2 cells, no cytotoxicity was observed which is in agreement with the assumption that certain CYP activities are crucial for estragole toxicity.

The major pathways of estragole metabolism include *O*-demethylation at the aromatic ring, epoxidation at the double bond of the allyl side chain, and 1'-hydroxylation at the allylic side-chain with subsequent enzymatic sulfation (Smith et al., 2002). The latter pathway leads to a semi-stable, reactive conjugate which can undergo sulfate elimination under formation of covalent bonds of the remaining moiety to nucleophilic targets, mainly in the liver (Phillips et al., 1981). The major enzymes catalyzing the initial step (1'-hydroxylation) are cytochromes P450 (CYP), most notably CYP1A2 (Jeurissen et al., 2007). There is evidence that the ratios between the major pathways change with dose, i.e., in rodents the percentage of the metabolite 1'-hydroxyestragole among the total urinary radioactivity decreased with decreasing dose (Anthony et al., 1987). The authors discussed that this trend was likely to be relevant in humans where background exposure to estragole via food etc. was estimated to be in the range of 10 µg per kg b.w. per day (Smith et al., 2002). Likewise, Zeller et al. (2009) found that only a very small proportion of estragole ingested with a fennel fruit infusion by seven test persons appears to be metabolized to the crucial 1´-hydroxyestragole. In a human volunteer, the percentage of 1'-hydroxyestragole excreted *via* urine declined with decreasing estragole exposure.

DNA adduct formation was considered as a hallmark of the liver carcinogenicity of estragole (Miller and Miller, 1983). In spite of its ability to covalently modify DNA bases, the

compound was classified based on dose-response considerations by Martins et al. (2012) as a 'weak' genotoxin and carcinogen. In fact, relatively high dose levels were required to cause DNA adducts and mutations in rodent liver (Suzuki et al. 2012a;b). Paini et al. (2010) tried to predict the formation of DNA adducts in the liver of male rats on the basis of *in vitro* incubations with pRH exposed to 1'-hydroxyestragole at concentrations between 0.5 and 150 μM. The model suggests that the formation of E3'N^2dG, the principal dG-adduct in rat liver, is linear up to a dose of at least 100 mg estragole/kg b.w. allowing the estimation of adduct yields at human background exposure levels. Later, Paini et al. (2012) reported a dose-dependent increase in the levels of E3'N^2dG in the liver of estragole-treated rats being statistically significant at and above 30 mg/kg b.w. It has to be noted, however, that the actual human exposure estimates, e.g., from herbal teas (0.5 mg estragole per day) and other food items are at least one order of magnitude lower (Smith et al., 2002).

Formation of the E3'N^2dG adduct has been demonstrated by Young et al. (2020) in pRH treated for 2 h with 50 μM estragole. The authors found about 100 adducts per 10^8 bases, i.e., a much lower adduct density than in the present study. The difference is probably due to the much lower time of exposure compared to our study where the highest adduct numbers were achieved after 12 - 48 h. In our study, estragole concentrations below 1 μM did not lead to significant adduct levels although an LOQ of one adduct in 10^8 bases was achieved. At 1 and 10 μM, a time-dependent increase in adduct levels was obtained, whereas at higher concentrations, a decrease was seen at later time points. This finding may be due to increased apoptosis and/or repair in adduct-bearing cells. The efficiency of repair of alkenylbenzene DNA adducts other than E3'N^2dG has not been studied in detail so far (Yang et al., 2020). In HepaRG cells, CHO cells, and pRH, no or very limited disappearance of E3'N^2dG was observed over several hours indicating marked persistence of the major adduct (Yang et al., 2020)'. The authors concluded from molecular dynamics simulations that conformational

changes in double-stranded DNA by the E3′N^{2}dG adduct are small, thus probably making it rather ineffective in triggering repair.'

From the data presented here, it is evident that the concentration-response characteristics for the levels of the two prominent estragole-derived adducts at dA and dG in pRH were linear when the whole concentration range between 0 and 300 μM was considered. It became hypolinear, however, when the modeling was focused on estragole concentrations ≤ 1 μM. There, the adduct levels were not different from the untreated controls and did not increase significantly with increasing substrate concentrations. This effect is due to the overweight of data at higher concentrations resulting in a relatively good fit to the linear model. In contrast, hypo-linearity of dose-response functions of genotoxic carcinogens is frequently observed in toxicology, in particular at very high and very low dose levels (Clewell et al., 2019). Such findings are probably due to a number of factors (Thomas et al., 2015) such as i) the level of DNA adducts depends not only on their formation *via* a reactive intermediate but also on the efficacy of their repair, ii) metabolic detoxification pathways, both enzyme-catalyzed or non-enzymatic, show a certain, limited capacity. Once being overwhelmed by increasing doses of a toxicant, the latter may get a chance to exert its toxicity to a full degree. It is currently not known if the lack of detectable adduct levels at low estragole concentrations is due to the aforementioned mechanisms.

There is good evidence that estragole is genotoxic in rat hepatocytes, e.g., in the UDS assay *in vitro* (Howes et al., 1990) or *ex vivo* in cells isolated form estragole-treated rats (Nesslany et al., 2010). The doses required for a positive response in the latter study, however, were at 250 mg/kg b.w. and above, i.e., at relatively high levels.

Here, we investigated micronuclei formation in human HepG2 cells and in the HepG2-CYP1A2 clone treated with estragole. Micronuclei formation was strictly dependent on the presence of CYP1A2. Furthermore, linear concentration-response characteristics were found for estragole concentrations between 1 and 300 μM. At 1 μM the micronuclei counts were still

statistically significantly different from the vehicle controls. Unfortunately, the relatively high variability in micronuclei counts, also between investigators, did not allow a reliable modeling of counts below 1 μM, which were not significantly, however, different from the vehicle control.

DNA adduct formation is considered as an important contributor to the genotoxicity of estragole. Since the liver is the principal target organ of carcinogenicity in mice and probably also in rats and estragole leads to metabolism-related DNA adducts in the target organ, it fulfills the criteria for a genotoxic carcinogen. It appears plausible to regard DNA adduct formation and damage as key initiating events in estragole carcinogenicity. However, our data and data by others provide evidence that the underlying dose (concentration)-response relationship in target cells is hypo-linear at the lower, more relevant end of the substrate concentrations. Thus the default assumption of dose-linearity of carcinogenicity of estragole strongly based on dose-response data at high concentrations/doses is probably inadequate, at least based on our *in vitro* findings, and needs to be replaced by a thorough dose-response analysis at relevant dose levels including additional *in vivo* studies.

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Figure legends

Fig. 1. Structural formulae, masses ([M+H]+), fragmentation pattern ($[M+H]^+$, and MS2-spectra) of (**A**) N^6-(isoestragole-3'-yl)-desoxyadenosine (E3'N^6dA), and (**B**) N^2-(isoestragole-3'-yl)-2'-desoxyguanosine (E3'N^2dG).

Fig. 2. Cytotoxicity of estragole (μM) in HepG2 cells (open bars) and HepG2-CYP1A2 cells (scattered bars) measured as percent resazurin reduction compared to vehicle (DMSO) only - treated controls. Cells were treated over 24 h and kept in culture for additional 72 before measurement. Bars represent means ± S.D. from n=3 independent experiments. **symbolize statistically significant differences from the control at $p \leq 0.01$.

Fig. 3. Adduct levels (adducts per 10^8 nucleosides) in rat hepatocytes in primary culture treated with vehicle (DMSO) only ('0') or various concentrations of estragole, over 6, 12, 24 or 48 h as indicated. Bars represent means ± S.D. for E3'N^6dA (**A**) or E3'N^2dG (**B**) from n=3 independent experiments. Asterisks symbolize statistically significant differences from the control at ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$; n.s. = non-significant.

Fig. 4. 'Broken stick' modeling of the concentration-response relationships between estragole concentrations and DNA adduct levels in rat hepatocytes in primary culture after 48 h of incubation. Data points represent all biological (n=3) and technical (n=3-4) replicates for E3'N^6dA **(A,B)** and E3'N^2dG (**C,D**) over the estragole concentration ranges between 0 and 300 μM (**A,C**) or between 0 and 1 μM (**B,D**).

Fig. 5. Micronuclei levels (micronuclei per 1,000 nuclei) in HepG2 cells (open bars) and HepG2-CYP1A2 cells (scattered bars) treated for 24 h with increasing concentrations of estragole, and a recovery phase of 72 h. Bars show means and S.D. of n=3 independent experiments. **symbolize statistically significant differences from the vehicle (DMSO) only –treated control (C) at $p \leq 0.01$.